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Isolating Mechanisms in Threespine Sticklebacks (*Gasterosteus*)[1]

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ABSTRACT

A systematic examination was made of isolating mechanisms, as set out by Mayr, that might serve to maintain reproductive isolation between the marine (*trachurus*) and the freshwater (*leiurus*) threespine sticklebacks. Field work was conducted in a small British Columbia coastal stream, the Little Campbell River, for 2½ years and complemented with laboratory experiments. Other streams were included late in the investigation. *Leiurus* permanently occupies the upper reaches of the stream; *trachurus* is anadromous and enters the lower reaches to breed in freshwater. Between the breeding grounds of the two, where numbers of both are greatly reduced, hybridization occurs. But it is restricted to a narrow zone.

The two species are easily distinguished, Thus, morphological analysis provided firm circumstantial evidence that hybrids are plentiful and that backcrossing occurs, predominately to *leiurus*. Hybridization was confirmed by rearing offspring under uniform conditions in the laboratory with crosses in all combinations. Such offspring were also used to demonstrate considerable genetic divergence (much of it polygenetic) between *leiurus* and *trachurus*.

Behavioural experiments demonstrated the absence of ethological isolation and hybrids performed courtship and parental care normally.

Nor was genetic incompatibility found in the reared hybrids (F_1's or backcrosses); all were vigourous. Seasonal isolation is only partially developed with early spawning migrants of *trachurus* making a major contribution to hybridization (in the Little Campbell River).

Since behavioural and genetic blocks to hybridization are not present, there is no means to prevent hybridization where *leiurus* and *trachurus* come together. However, coexistence between the two species is very low. Evidence from observation and experiment in the field and from preference tests showed that ecological isolation is a very powerful barrier to hybridization. The two species show numerous adaptations to the distinctly different habitats they frequent, and each shows a strong affinity for its own habitat. In localities with intermediate or contiguous habitats, coexistence and interbreeding occur. Hybridization is a function of the environment.

No selection against hybrids could be detected within the hybrid zone (or with laboratory reared hybrids); yet, one is forced to assume that it is present outside the zone. The very narrow zones as well as the reversed cline that were found indicate there is intense selection against hybrids. What these selective forces are remains to be found. Hybrid zones will probably continue to be poorly understood until a critical analysis of hybrid inferiority is made.

Genotypes of either species that remain in the hybrid zone are at a strong selective disadvantage. Hence, reinforcement of ecological isolation probably occurs, and Moore's criticism concerning the spread of such reinforced genotypes would not apply to such cases. Mayr distinguishes between pre- and postmating mechanisms stating that the mode of operation of natural selection will be different for the two. But in threespine sticklebacks one premating mechanism (ecological isolation) and one postmating mechanism (hybrid inferiority) cannot be distinguished. This is so because ecological isolation is the cause of hybrid inferiority.

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Leiurus and *trachurus* are reproductively isolated, have well developed isolating mechanisms, and exhibit considerable genetic divergence. The two, then, fulfill the species definition of Mayr. There is no evidence that introgression occurs. Indeed a reversed cline showing a progressive increase in morphological divergence between the two species as the hybrid zone is approached together with the narrow hybrid zone demonstrates that selection severely restricts gene flow. Collections and observations from other streams corroborate those from the study area. Reproductive isolation between *leiurus* and *trachurus* seems to be widespread, throughout their range.

INTRODUCTION

THE DIVERSITY of life springs from isolating mechanisms, a fact recognized and stressed by Dobzhansky (1937), Huxley (1942), and Mayr (1942). It is now firmly established that isolating mechanisms are among the most important attributes of species. Their origin, interaction, and mode of operation are a vital field of study. Detailed analyses of mechanisms that prevent interbreeding between closely related sympatric species are particularly critical, for such studies are essential to understanding the origin of species (an issue Darwin bypassed).

The threespine stickleback (*Gasterosteus aculeatus* L.) offers exceptional opportunity for investigation. This small fish is widespread and abundant in boreal regions, and is easy to rear and to maintain in the laboratory. Furthermore, there is already considerable information available for this species. Of the two forms now recognized, one is marine but migrates into freshwater to breed, and the other inhabits freshwater permanently. Since the two are often sympatric during the breeding season, one is led to ask how genetic isolation is maintained.

There are a host of mechanisms that may isolate closely related species, and several classifications have been proposed. The following classification of Mayr (1963) was adopted in this investigation:

1. Mechanisms that prevent interspecific crosses (premating mechanisms).
   (*a*) Potential mates do not meet (seasonal and habitat isolation).
   (*b*) Potential mates meet but do not mate (ethological isolation).
   (*c*) Copulation attempted but no transfer of sperm takes place (mechanical isolation).
2. Mechanisms that reduce full success of interspecific crosses (postmating mechanisms).
   (*a*) Sperm transfer takes place but egg is not fertilized (gametic mortality).
   (*b*) Egg is fertilized but zygote dies (zygote mortality).
   (*c*) Zygote produces an F_1 hybrid of reduced viability (hybrid inviability).
   (*d*) F_1 hybrid zygote is fully viable but partially or completely sterile, or produces deficient F_2 (hybrid sterility).

Sticklebacks are external fertilizers so that mechanical isolation can be omitted from further consideration.

Very few attempts have been made to systematically determine which of the potential isolating mechanisms function for a given pair of species. The objective of this research was to find out which of the above mechanisms, if any, separate the marine and freshwater forms of the threespine stickleback, and to determine the relative importance and interplay among the isolating mechanisms. The methods used were those of observation and experiment in the field and in the laboratory.

As an introduction to the investigation it is necessary to outline the life history of threespine sticklebacks. The distribution is nearly circumpolar, *Gasterosteus* being absent only from the Arctic Siberian coast. It occurs as far south as Baja California (in freshwater) and the Mediterranean Sea (Andriyashev, 1964). Although landlocked populations of the marine form are known (Munzing, 1963), the freshwater form is unknown from the sea. Following Munzing and others the marine form is referred to as *trachurus*, and the freshwater form is called *leiurus*. *Trachurus* lives in the sea for most of its life, migrating into freshwater in the spring to breed. In early autumn the offspring and adults return to the sea. The life span of this form is probably 1 year (van Mullem and van der Vlugt, 1964). *Trachurus* is distinguished by a host of features, but may readily be identified by a complete series of bony plates along its sides, its large size, and a bright silver colour (Heuts, 1947b; Munzing, 1963). The freshwater form, *leiurus*, remains in freshwater streams or ponds throughout its life. The life span may be about 2 years (Greenbank and Nelson, 1959) but this is not well established (van Mullem and van der Vlugt, 1964). It has few bony plates on its sides, a small size, and an olive colour mottled with indistinct bars.

The males of both forms assume brilliant nuptial colours with red throats and blue irises. During the breeding season they are very aggressive and establish territories, build nests in the substrate, guard and fan their eggs, and exhibit parental care of fry. Their elaborate courtship and reproductive activities are well known; and for detailed descriptions and further references the reader is referred to the work of van Iersel (1953).

THE STUDY AREA

General Materials and Methods

An intensive field study began at the Little Campbell River in 1964 and continued through the summer of 1966. Several days were spent each week at the study area from March through September, but during the winter, visits averaged twice a month. Three localities were chosen for detailed studies: Station D, a representative *leiurus* habitat; Station N, a representative *trachurus* habitat; and Stations L, M, and M′, which represent the hybrid zone.

Salinities were recorded with a set of three hydrometers registering densities of 1.000–1.011, 1.010–1.021, and 1.020–1.031 specific gravity of NaCl. During the breeding season a Buchler–Cotlove chloridometer was also used to determine salinity of water from the *trachurus* breeding grounds (Station N). These measurements were compared with recordings from the upper reaches of the stream

to detect small differences in salinity that might exist as a result of tidal flux. Total dissolved solids were determined with an r-c conductivity bridge. Water samples for *p*H measurements were collected from the stream and immediately taken to the laboratory for determination with a Beckman *p*H meter. Stream currents were measured with a Gurly current meter or a table-tennis ball floated on the surface over a known distance. The latter method was used for measuring average conditions over a large section of the stream.

Fish were collected with a 3 m × 2 m seine of woven nylon with 3-mm stretched mesh, and the lead line was heavily weighted to facilitate collecting in dense vegetation. Where seining was impossible, a variety of fine meshed dip nets were used. Live fish were transported in 8-gal styrofoam coolers, and collections for morphological studies were preserved in 15% buffered formalin. Invertebrates were preserved in 80% ethyl alcohol.

The Habitats

A small coastal stream, the Little Campbell River, was chosen for a detailed study of *Gasterosteus* because it is readily accessible and because *leiurus* and *trachurus* are abundant there. It is located in the extreme southwest corner of British Columbia and empties into Semiahmoo Bay at White Rock (Fig. 1).

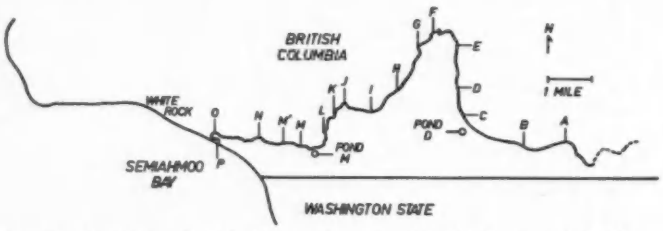

Fig. 1. Locations of collecting stations on the Little Campbell River.

The Little Campbell River is approximately 16½ miles long. The width varies from less than 10 ft in the upper reaches to more than 36 ft at the estuary. For most of the length it is easily waded, but isolated pools and stretches of channels are as deep as 7 ft. The water level fluctuates considerably. During the spring, intermittent flooding occurs so that meadows along the banks are covered with thin sheets of water, particularly in the upper reaches. On the other hand, the water level drops very low in summer and current may be scarcely noticeable. The narrow width and shallow waters are suitable for efficient seining and field studies.

Major habitats and their features are shown in Table I. The headwaters (Stations A–E) are approximately 7½ miles long and lie at an altitude of 220 ft. The entire area is marshy with a mud bottom and dense aquatic vegetation that chokes sections of the stream in the summer. *Leiurus* is extremely abundant, and density estimates shown in Table II convey some idea of their numbers. Ponds along the stream also have dense populations of *leiurus*. This form

Table I. A synopsis of habitats found in the Little Campbell River. Physical and chemical data represent average conditions during the breeding seasons of *leiurus* and *trachurus*.

Habitat

TABLE 1. A synopsis of habitats found in the Little Campbell River. Physical and chemical data represent average conditions during the breeding seasons of *leiurus* and *trachurus*.

	Headwaters (*leiurus* habitat) (A–E)	Riffle section (E–K)	Habitat hybrid zone Station (L–M′)	Lower reaches (*trachurus* habitat) (N)	Estuary (O–P)
Stream gradient	8 ft/mile	32 ft/mile	12 ft/mile	11 ft/mile	
Water velocity	3 cm/second	74 cm/second	23 cm/second	10 cm/second	10 cm/second
Temperature	16 C	14 C	15 C	15 C	16 C
Salinity	0.18‰	0.18‰	0.20‰	0.21‰	Mixohaline (0.17‰–12‰)
Total dissolved solids	65 ppm	–	118 ppm	118 ppm	–
*p*H	6.8	7.1	7.8	7.8	–
Water colour	Medium tea colour; slightly turbid	Clear	Light tea colour to clear; slightly turbid	Colourless; slightly turbid	Turbid
Bottom	Soft and muddy	Rocks and rubble	Intermingled mud, medium grained sand, and some silt	Sand	Mud, sand, silt, clay, and some oyster beds
Vegetation	Dense beds of *Nuphar*, *Myosotis*, *Oenanthe*, *Glyceria*, *Carex*, *Typha*, *Potamogeton*; also *Lemna* and green algae	Very sparse; some encrusting algae (*Cladophora*)	Some *Elodea* and *Myriophlyllum*; some *Glyceria*, *Carex*, *Typha*	Dense beds of *Elodea*	Dense beds of *Ulva* and some *Enteromorpha*
Vertebrates Fish:	*Catostomus catostomus*, *Cottus asper*, parr of *Oncorhynchus kisutch* and *Salmo clarki*	*C. asper*, *C. aleuticus*, *O. kisutch*, *S. clarki*	*C. asper*, *C. aleuticus*,	*C. asper*, *C. aleuticus*, *O. kisutch*, *Platichthys stellatus*	*C. asper*, *C. aleuticus*, *P. stellatus*, *Oligocottus maculosus*, *Leptocottus armatus*
Amphibians:	*Bufo boreas*, *Rana pretiosa*, *Hyla regilla*, *Ambystoma gracile*	*B. boreas*	*R. pretiosa*		
Invertebrates Molluscs:	*Lymnaea*, *Physa*, *Ancylus*, *Musculium*, *Margaritifera*, *Gyraulus*	*Ancylus*		*Musculium*, *Pisidium*, *Margaritifera*	*Crassostrea*
Crustaceans & annelids:	*Amphipods and* leeches abundant			*Gammaridae*, *Palaemonetes*	*Balanus*, *Gammaridae*, *Palaemonetes*
Aquatic insects:	Naiads of dragon and damselflies, *Sialis*, *Nepa*, Chironomidae, Chrysomelidae, Ephemeroptera	Trichoptera, Ephemeroptera, Plecoptera		Trichoptera, Chrysomelidae, Chironomidae, Dixidae, naiads of damselflies	

tends to congregate in shallow pockets along the margins of the stream where vegetation is most dense and is less numerous where the banks are steep and the vegetation is sparse. Such a distribution gives rise to marked local variations in density (Table II). In the spring, males with breeding colours can be found all over the flooded meadows.

TABLE II. Density estimates for *leiurus* and *trachurus* from several stations in the Little Campbell River. Each figure represents the average catch from three adjacent 15-ft seine hauls. Consecutive figures for a given date are similar estimates made at 200-ft intervals of stream. Densities of *leiurus* in 1965, not given, were like those for 1964.

Leiurus, 1964

Station D				Station K		Station M	
March 6	7	May 4	123	March 16	8	March 16	7
	112		216		22		3
	97				10		15
March 13	138	May 28	416	April 18	17	April 17	0
	101		28		5		3
	86		312		6		1
April 5	116	June 9	432	May 3	19	May 3	5
	204		156		3		16
	9		82		9		8
April 23	27	June 22	257	June 12	6	June 12	11
	14		46		31		1
	196		315		14		5

Trachurus

1964			1965			
Station N		Station O	Station N			
June 16	212	9	June 8	43	July 1	9
	193	3		21		5
	247			23		
June 23	184	2	June 18	12	July 5	6
	202	8		8		7
		7		16		
July 6	164	5	June 22	11	July 14	12
	221	3		20		3
	202	5				8
July 21	175	26	June 26	19	July 20	7
	124	7		6		5
	256					12
August 8	167	3				
	206	11				
	198	2				

The headwaters habitat gives way abruptly (within 150 ft) at Station E, where the riffle habitat (Stations E–K) begins, and extends for the next 4½ miles. This section is characterized by swift water, rocky bottom, lack of vegetation, and virtually no sticklebacks.

At Station K there is again a rapid transition from the riffle habitat to one more like the headwaters. But there is less vegetation, slightly faster current, and some sand and silt. *Leiurus* is less abundant here than in the upper reaches (Table II). A short distance downstream is a section called the hybrid zone for reasons to be discussed later. This section of the stream is one mile long, and Table I shows that for features such as bottom type, vegetation, and water quality, it is intermediate between the headwaters habitat and the lower reaches.

The lower reaches (Station N) occupy ¾ mile and are 1½ miles from the estuary. The habitat has a sandy bottom, a mild current, and dense beds of *Elodea*. This is the only aquatic vegetation present, which covers most of the bottom in the summer but dies away in the winter. When *trachurus* migrates into the stream, in late spring, it settles to breed at Station N. Salinity measurements made throughout the breeding season with hydrometer and chloridometer show that the water is fresh and that it does not differ much in salinity from water in the upper reaches (Table I). Table II indicates the large numbers of *trachurus* that breed in this habitat. *Trachurus* associates closely with *Elodea*, and since *Elodea* is uniformly distributed, this probably accounts for the more uniform distribution of *trachurus* numbers along the stream when that form is compared with *leiurus* (Table II).

The data also demonstrate a striking decrease in numbers found in 1965. *Trachurus* spawning grounds are concentrated into a short section of the stream and this habitat was intensively seined in 1964. The heavily weighted lead line of the seine probably destroyed many nests and fry. The decrease in density found in 1965 suggests that *trachurus* homes to a given stream to spawn since many of the fry that would have returned to the stream in 1965 may have been killed in 1964.

The estuary (Stations O–P) is an unstable habitat with large fluctuations in water level, mixohaline water, and dense beds of *Ulva*. Small numbers of *trachurus* can be found throughout the summer (Table II).

MORPHOLOGICAL ANALYSIS

Materials and Methods

Leiurus and *trachurus* differ in a number of morphological characters. Those chosen for analysis were number of lateral plates, number of gill rakers, electrophoretic patterns, standard length at sexual maturity, body depth into standard length at sexual maturity, and numbers of dorsal and anal rays. These characters were selected because they could be most easily and objectively measured, and because the two forms showed conspicuous differences in those characters. Other differences, for example number of vertebrae, length and shape of dorsal and pelvic spines, and colour patterns, were present but were not used.

Procedures for counts and measurements were in most instances those suggested by Hubbs and Lagler (1958). The exceptions were counts of lateral bony plates and electrophoretic analysis. All lateral plates were counted including the small anterior ones and those in the caudal keel. Counts were made using a binocular dissecting microscope and a probe. Fish smaller than about 3 cm were stained with a few crystals of alizarin dye in 2% KOH for 24 hr before plates were counted, to render the small anterior and posterior plates conspicuous. Dr H. Tsuyuki at the Fisheries Research Board of Canada Technological Station, Vancouver, did the electrophoretic analysis on muscle protein using previously published methods (Tsuyuki and Roberts, 1962). Only sexually mature fish were used to measure standard length and body depth into standard length so that a more uniform comparison among the parental forms and hybrids could be made. Vernier calipers were used to make all measurements. Sticklebacks used in the morphological analysis and as parents to obtain progeny were collected from the Little Campbell River during the spring and summer of 1964 and 1965.

Individuals of both forms were found together at Stations L, M, and M′ during the breeding season along with numerous variable and intermediate individuals. Samples from this section of the stream were used to determine the presence of hybridization. F_1 hybrids in fish are almost always intermediate between the parental types in their morphological features (Hubbs, 1955), and the presence of such individuals together with both parental types provides strong circumstantial evidence for hybridization. Moreover, the presence of individuals that approach one or the other parent more closely in their morphology suggests that backcrossing has occurred (Hubbs, 1955). Thus, once the morphology for the characters indicated is determined for allopatric populations, a comparison with samples from the zone of overlap between *leiurus* and *trachurus* may indicate the presence of hybridization and backcrossing.

Care was taken to sample the hybrid zone uniformly by seining for fish over the entire distance from Station L to Station M′. Although both parental types are present in the hybrid zone, only hybrids are included in this analysis. Hybrids were identified by their plate counts, which included all sticklebacks with counts intermediate to those obtained from allopatric populations of each form (i.e. with counts over 7 and under 30). However, the data set out in Fig. 6 and 7 are based on morphological characteristics for all sticklebacks throughout the length of the stream. Also, when samples were collected to provide material for constructing a hybrid index, all sticklebacks taken in the hybrid zone were included. Data for the morphological analyses of sympatric populations (hybrids) are presented along with data for allopatric populations so that comparisons can be most easily made.

Allopatric Populations

In order to differentiate the morphology of *leiurus* and *trachurus* samples were collected from allopatric populations of each. These samples assure, as

much as possible, against the effects of hybridization. Furthermore, they serve as controls so that once their morphological characteristics are known, the samples can be compared with sympatric populations of *leiurus* and *trachurus*.

Samples from populations of *leiurus* were taken from the headwaters at Stations A, B, C, and D, and from populations of *trachurus* at Station P. It may be reasonably assumed, as I hope to demonstrate, that these populations are allopatric.

Lateral Plates.— Figure 2 demonstrates the great utility of plate counts as a ready means of identifying the two forms. There is wide divergence and no overlap in counts, 3–7 plates being diagnostic for *leiurus* and 30–35 plates being diagnostic for *trachurus*.

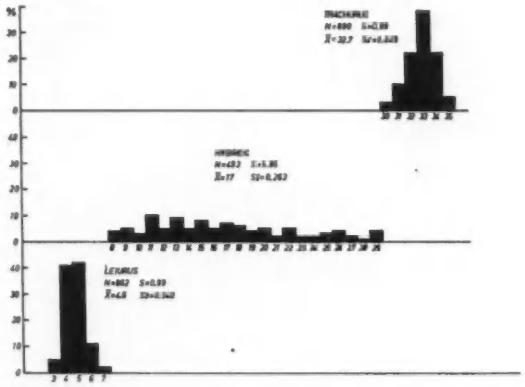

FIG. 2. Plate counts (abscissa) for allopatric populations of *leiurus* and *trachurus* and for hybrids. N, sample size; $\bar{X}$, mean; *s*, standard deviation; $s\bar{x}$, standard error.

Gill Rakers.— This character provides a useful means of distinguishing the two forms (Fig. 3), *leiurus* averaging considerably fewer rakers than *trachurus*. There is an overlap of only 4% in counts, which was determined by estimating the per cent of individuals in that form with least numbers of individuals in the overlapping zone.

Electrophoretic Patterns.—Like plates this character can be used to identify each form because there are bands that are unique to each (Fig. 4).

Standard Length of Adults.— Populations of *leiurus* and *trachurus* may be differentiated by adult length (nuptially coloured males and gravid females), *leiurus* having a smaller average size than *trachurus*. However, an overlap of 50% makes this character less useful than others (Fig. 5).

Body Depth into Standard Length.— *Leiurus* has a deeper body and *trachurus* a more terete body (Fig. 5). For this character there is an overlap of 14% between forms.

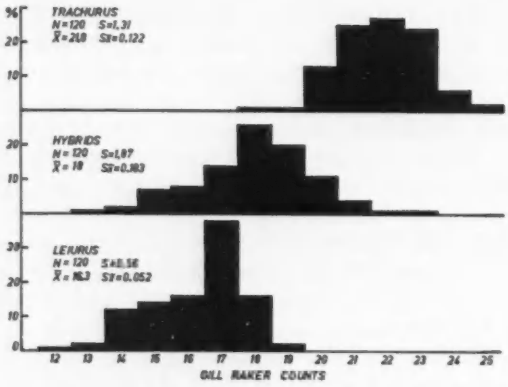

FIG. 3. Gill-raker counts for allopatric populations of *leiurus* and *trachurus* and for hybrids.

Dorsal and Anal Rays.— *Leiurus* and *trachurus* differ in dorsal and anal ray counts (Fig. 5), and in each character *leiurus* averages fewer rays than does *trachurus*. Overlap in dorsal rays is 36% and in anal rays is 31%.

THE HYBRID ZONE

Hybrids show a complete range of plate counts that are intermediate between the parental types (Fig. 2). Such a large amount of variation for a character so divergent in the parental forms suggests that backcrossing takes place. The predominance of counts approaching the *leiurus* parent further suggests that the majority of backcrosses are to that form.

Inspection of data for other morphological features of hybrids shows a pattern similar to that obtained from plate counts (Fig. 3, 4, and 5) in that hybrids are always more or less intermediate in counts and measurements. Frequency distributions for all characters but dorsal rays are skewed toward *leiurus* suggesting considerable backcrossing to that parent (percentages of hybrid classes obtained from electrophoretic analysis are set out in Fig. 4).

If the hybrid zone is divided in half between Stations M and M′, and the hybrids are classed on the basis of plate counts as either more similar to *leiurus* (counts from 8 to 16) or more similar to *trachurus* (counts from 21 to 29), then the numbers in these hybrid classes (called backcrosses) for the upper and lower halves of the hybrid zone are, respectively: for *leiurus* backcrosses, 132 and 38, for *trachurus* backcrosses, 41 and 76. When the data are tested with a contigency table a value of $P < .005$ is obtained indicating that hybrids are not randomly distributed. Backcrosses to *leiurus* are mostly in the upper half of the hybrid zone near Station K, and backcrosses to *trachurus* are mostly in the lower half of the hybrid zone nearest Station N.

The outstanding features of the hybrid zone are its narrowness, its sharply defined boundaries, and the intermediacy of its ecological properties between

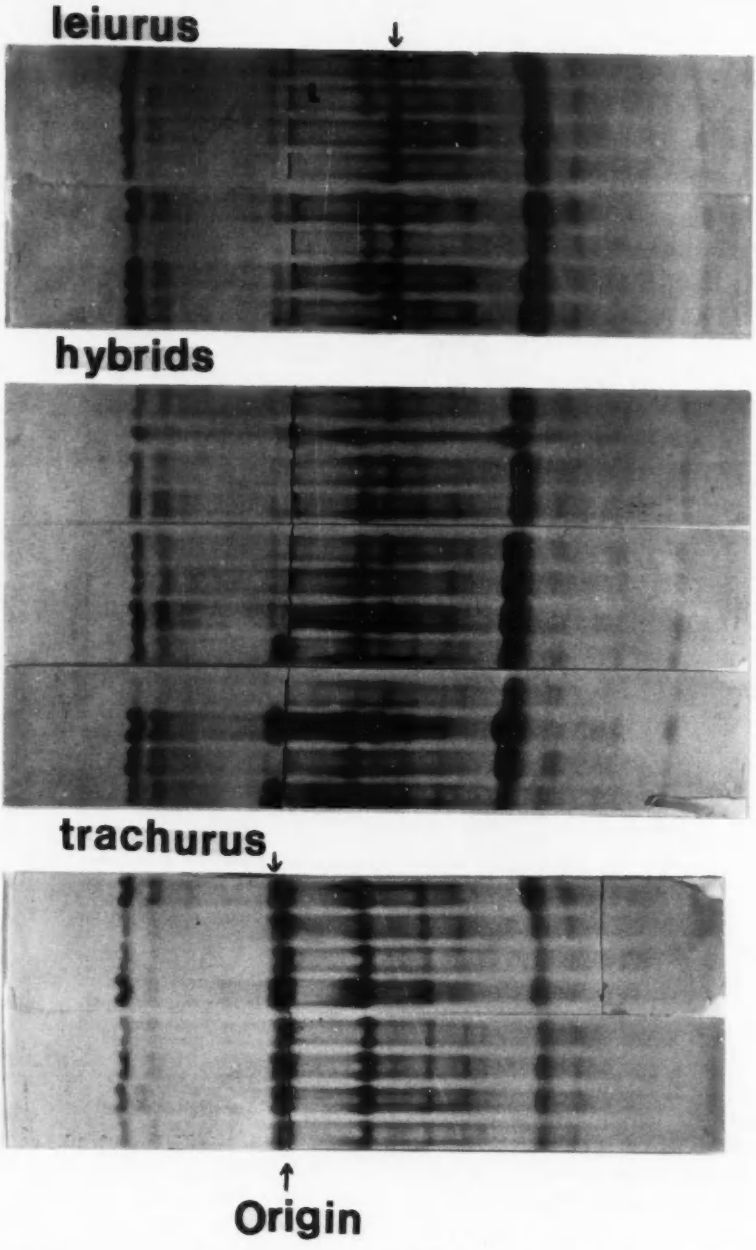

FIG. 4. Electrophoretic patterns of muscle protein for allopatric populations of *leiurus*, *trachurus*, and hybrids. Diagnostic band of each form is indicated by an unlabeled arrow. Sample sizes are 60 *leiurus*, 60 *trachurus*, 120 hybrids. Hybrids have either both bands diagnostic of each form or only one hand diagnostic for one or the other form. These are interpreted as either F_1's or one or the other backcross class. Percentages for the three classes are: 11.8% F_1; 80.2% backcross to *leiurus*; 8% backcross to *trachurus*.

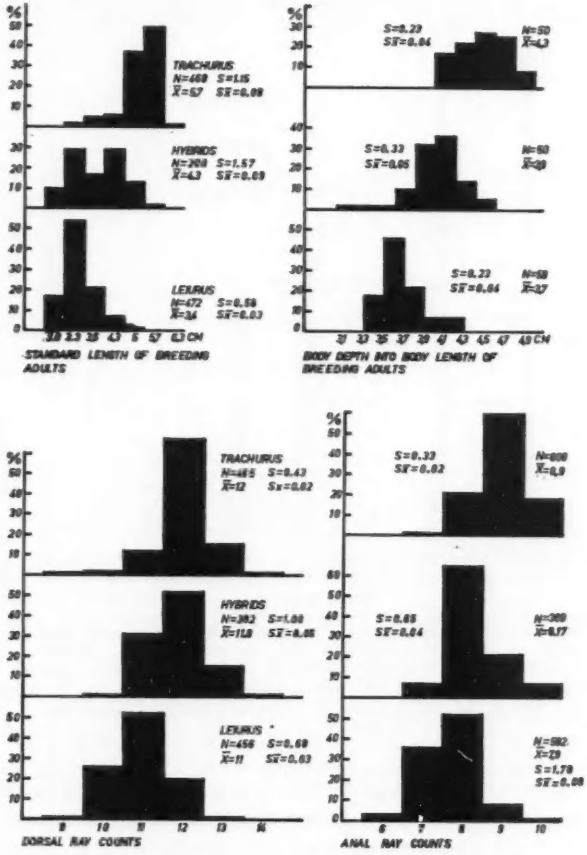

FIG. 5. Morphological characteristics for allopatric populations of *leiurus*, *trachurus*, and hybrids.

those of the headwaters (*leiurus* habitat) and of the lower reaches (*trachurus* habitat).

When plate counts of samples from each station are plotted against distance from the sea throughout the length of the stream (Fig. 6), it is clear that a continuous cline does not exist. On the contrary, there is a well-defined zone of intergradation between the parental forms. Moreover, when samples of sticklebacks at each station are analyzed for all morphological characteristics used above, except electrophoretic patterns, and identified accordingly as hybrids, *leiurus*, or *trachurus*, one again finds a well-defined zone of intergradation (Fig. 7). In each instance it is precisely in this zone that reduced numbers of both parental forms occur, together with variably intermediate

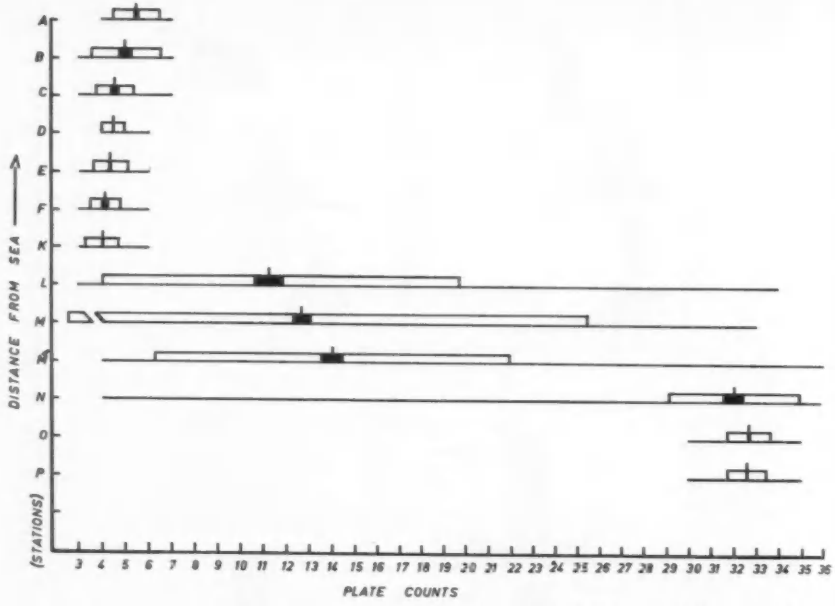

FIG. 6. Plate counts for sticklebacks at each station along the Little Campbell River. Minimum sample size for any station is 237 individuals. The range of variation is shown by the heavy horizontal line; the mean by a small vertical line. Each black bar represents two standard errors of the mean on either side of the mean; one-half of each black bar plus the white bar at either end shows one standard deviation on either side of the mean.

individuals. In the autumn after *trachurus* has migrated to the sea, the structure of either of the above graphs is completely altered since the entire left side would, of course, be represented as a blank space.

If plate counts for *leiurus* only are examined (Fig. 6) there exists an obvious cline for this character. Suprisingly, the direction of the cline is such that mean counts increase with distance upstream so that highest counts are in the headwaters and lowest counts are nearest the hybrid zone at Station F. The direction of the cline is, then, the reverse to that expected if introgression from the hybrid zone were affecting the character. This demonstrates not only the absence of introgression for that character, but also strong counter-selection against genetic exchange between *leiurus* and *trachurus*. The cline can be seen in detail in Fig. 8.

Thousands of individuals were examined in the headwaters but not one *trachurus* or hybrid was found, and neither *leiurus* nor hybrids were found at Station P. This, along with the evidence presented above, would seem adequate to justify the assumption that the populations concerned are allopatric.

It should be noted that mixed breeding populations of *leiurus* and *trachurus* do not occur in the hybrid zone (Fig. 7). The numbers of *leiurus* are much

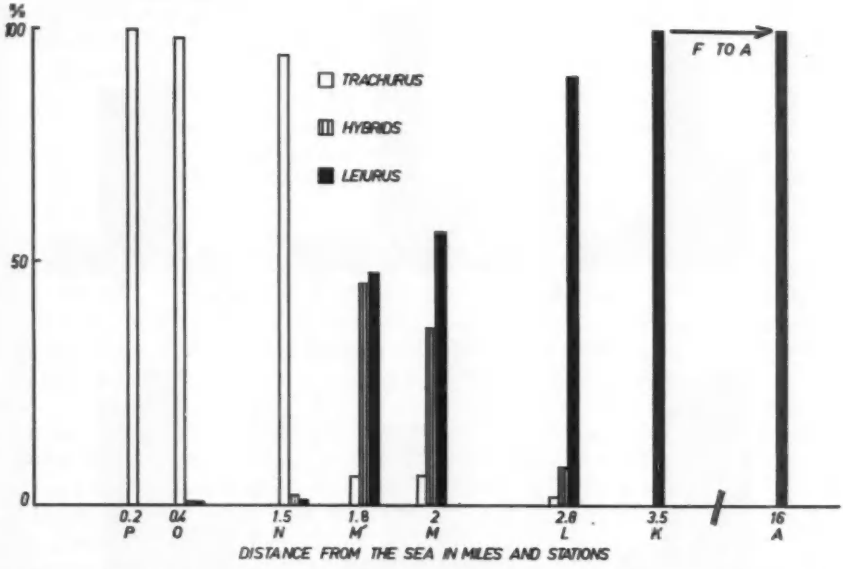

FIG. 7. Distribution of *leiurus*, *trachurus*, and hybrids at each station along the Little Campbell River. Minimum sample size for any station is 237 individuals.

smaller there than in the headwaters (Table II), and only occasional individuals of *trachurus* are captured. At Stations M and M′, where the largest numbers of *leiurus* and *trachurus* are caught together, hybrid swarms have developed. The proportions of *leiurus*, hybrids, and *trachurus*, are 10:8:1. At Station L the numbers of *leiurus* increase whereas the numbers of hybrids and of *trachurus* decrease (Fig. 7). Hybrids and *leiurus*, are also common in a pond adjacent to the stream (Pond M in Fig. 1), but *trachurus* is rare.

THE HYBRID INDEX

Once the presence of hybrids is demonstrated, the possibility of introgression exists. As introgression can have profound and far reaching effects (Anderson, 1949; Stebbins, 1959), a critical examination is necessary. If one attempts to establish the presence of introgression using any single character, the results may be misleading for a number of reasons. One needs only to consider the genetic basis — dominance, oppositional effects, and other interactions of genotypes — to realize this. More often effects of recombination make the use of one character less reliable. For example, plates can be reliably used to identify a hybrid but it was found that in laboratory-reared fish a few hybrids cannot be recognized as such using plates alone. Backcrossing and recombination yield some offspring with counts like those of the parent form, and such hybrids cannot be identified using plate counts only, but raker counts

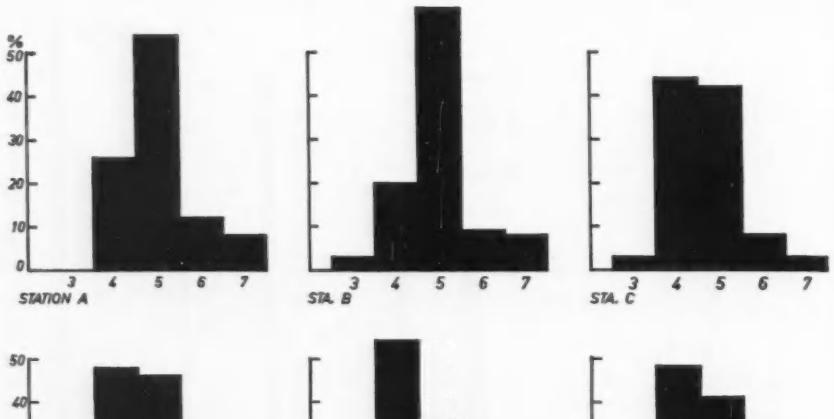

FIG. 8. Clinal variation in plate numbers of *leiurus*. Minimum sample size for any station is 237 individuals.

reveal their hybrid origin. And so it is for any single character, so that combining a number of characters into a single index and weighting each relative to its usefulness, one has a more powerful tool to detect introgression and its direction.

Fresh samples of 40 *leiurus* and 40 *trachurus* were collected from allopatric populations along with 144 individuals taken throughout the hybrid zone. These were taken to the laboratory alive, and once counts and measurements were made, the fish were immediately frozen in preparation for electrophoretic analysis of muscle protein. Each individual was analyzed for five morphological features. Dorsal and anal ray counts were excluded from the index because they were found useless (see Laboratory-Reared Fish).

The well-known method of Anderson (1949) for constructing a hybrid index has great utility to detect hybridization and introgression, and was used in this analysis. The five characters used in the index were selected because of their demonstrated success in identifying the two forms and hybrids.

CLASSING AND SCORING

The rationale behind assigning classes and scores to characters is the following. Any character that is widely divergent between the two forms is divided into more classes than a less divergent character. This is so because

there can be a wider spectrum of variation expressed in the hybrids, thus allowing intermediate counts or measurements (F_1 hybrids), and counts or measurements approaching one or the other parent (backcrosses), to be realized. If for other characters there is less divergence, less variation can be expressed in hybrids. Consequently, fewer classes must be used. When much overlap exists in a character, only three classes can be assigned, one for each form and one for intermediates. And, if overlap is present, only the extreme counts of each form (counts outside the region of overlap) are grouped as a class. This produces a more efficient separation of the two forms from hybrids, and of the forms from one another.

Scores are assigned in proportion to the usefulness of a character. If the character is objectively determined, diagnostic for *leiurus*, *trachurus*, and hybrids, and with no or little overlap, it is assigned a maximum score. As these criteria diminish, or overlap increases, the character is scored less.

After all characters have been analyzed for an individual and the scores for the five characters determined they are added together to give a hybrid index value for the specimen. In this way indices are obtained for all individuals. Then hybrid index values for all specimens are grouped for presentation in a frequency distribution. This identical procedure is carried out independently for allopatric and sympatric populations so that the two can be compared and, thus, the existence and direction of introgression evaluated. Finally, one form (*leiurus*) always receives minimal scores of zero, and the other form always receives maximal scores for each character.

Grouping and assigning of classes are not entirely arbitrary. The groupings of plates, or any other character, for classes is determined by the range of variation encountered in allopatric populations of *leiurus* and *trachurus*. In this instance, allopatric populations of *leiurus* have plate counts ranging from 3 to 7, and for *trachurus*, from 30 to 35. Thus, for *leiurus*, counts from 3 to 7 must be grouped as a class, and for *trachurus*, counts from 30 to 35 must be grouped for another. Although grouping of intermediate counts for classes is arbitrary, the bias is more apparent than real. For example, counts from 8 to 12 and 13 to 16 are more similar to *leiurus* than to *trachurus*. This is so whether they are lumped together as one class, split as two classes, or split for more. Scoring a character is certainly arbitrary, but again the bias is more apparent than real. I found that plates are most useful to identify *leiurus*, *trachurus*, and hybrids. This being so, plates must be scored most heavily. Equally, the character that is least useful for identifying the two forms (length in this instance) must be scored least, and so contributes least to the total index. Finally, any bias resulting from scoring is minimized since allopatric and sympatric populations are treated in an identical manner, and since the two are only compared for degree of difference.

Lateral Plates.— This character was given seven classes, one each for the following groups of plate counts: 3–7 (*leiurus*), 30–35 (*trachurus*), 17–20 (*intermediate*), 8–12 and 13–16 (approaching *leiurus*), 21–24, and 25–29 (approaching *trachurus*). The maximum score of 12 was assigned to plates because it is

objectively measured, as all characters used are, it is diagnostic for *leiurus*, *trachurus*, and hybrids, and is widely divergent with no overlap.

Gill Rakers.— Five classes were assigned to raker counts: 12–14 (*leiurus*), 23–25 (*trachurus*), 18–19 (intermediate), 15–17 (approaching *leiurus*), 20–22 (approaching *trachurus*). For this character and the remaining ones, excepting electrophoretic patterns, only the extreme counts of *leiurus* and *trachurus* were grouped for reasons discussed above. Rakers fulfill the criteria for maximal scoring but for a small overlap of 4%. Accordingly they were scored less than plates, with a value of 10.

Electrophoretic Patterns.— Only three classes could be assigned this character since divergence is small. The one band diagnostic of *leiurus* received a class, the other diagnostic of *trachurus* received a second, and individuals with both present received a third. However, banding patterns fulfill the remaining criteria and so were scored 10, for although they are less divergent than rakers, there is no overlap.

Standard Length of Adults.— Sizes were divided into only three classes because of the 50% overlap between the forms. Classes were: 3–3.5 (*leiurus*), 5.4–6.3 (*trachurus*), 3.6–5.3 (intermediates). A minimal score of 4 was assigned to size because of considerable overlap.

Body Depth into Standard Length.— Classes were as follows: 3.1–3.5 (*leiurus*), 4.5–4.9 (*trachurus*), 3.6–4.4 (intermediates). The character was scored 6. The 14% overlap warrants a heavier score than size but less than banding patterns or rakers.

Scores for the five characters were summed for every individual. *Trachurus* always received maximum scores so that the highest index possible was 12 + 10 + 10 + 6 + 4 = 42. *Leiurus* received minimum values for all characters so the lowest index that could be obtained was zero.

A comparison between indices compiled for allopatric and sympatric populations shows that *leiurus* and *trachurus* from allopatric populations are well separated (Fig. 9). However, the integrity of the two forms breaks down in

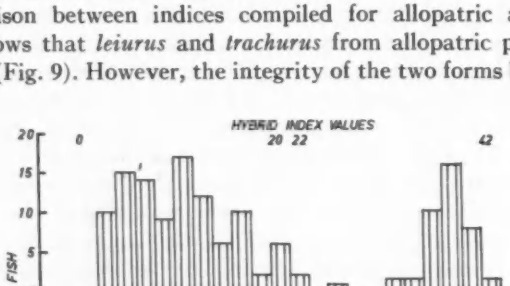

FIG. 9. The hybrid index; inverted histograms represent allopatric populations, and upright histograms represent samples from the hybrid zone.

the hybrid zone. They are no longer separated by a well-defined gap, the morphological features of *leiurus* becoming blurred with those of *trachurus*. It is also obvious that *leiurus* is more affected than *trachurus*, presumably as a result of gene flow. The absence of a distinct intermediate mode suggests that F_1 hybrids are relatively few, and the scarcity of individuals approaching *trachurus* indicates that backcrosses to that form are rare. Results from laboratory-reared fish support these interpretations.

Laboratory-Reared Fish

To confirm the presence of hybrids found in the wild the following classes of progeny were artifically produced and reared under uniform conditions in the laboratory: *leiurus* offspring, *trachurus* offspring, reciprocal F_1 hybrid offspring, reciprocal backcrosses to *leiurus* and to *trachurus*. Parents used to obtain eggs and sperm for *leiurus* and *trachurus* progeny were taken from the allopatric populations mentioned earlier, and as well, provided parental stocks for the production of reciprocal F_1 hybrid offspring. Backcrosses were made by first collecting presumed F_1 hybrid parents from the hybrid zone. These individuals were identified by their plate counts, and only individuals that were strictly intermediate between the two forms were used. One cannot be certain that such individuals are F_1 hybrids, but evidence presented in another section (Seasonal Isolation) shows that hybrids are largely prevented from interbreeding by differences in breeding seasons of the sexes. The possibility of an intermediate hybrid being an F_2 is, therefore, unlikely. Backcrosses were then made by crossing putative F_1 hybrid parents with parents of each form in the four reciprocal combinations. Plate counts of parental stocks are given in Fig. 10.

Artificial fertilization was produced by stripping eggs into a small moist petri dish. A male's testes were removed and placed in a watch glass with a few drops of tap water, chopped into fine pieces with forceps and probe, and poured over the eggs as the container was gently rotated. Eggs were left to harden for 20 min and were then observed through a binocular dissecting microscope. This precaution was taken to ensure that the perivitelline membrane had separated from the egg (an indication of successful fertilization). Mucous around the egg mass was removed by placing the tip of a dropper into the centre of the mass and pressing it against the bottom of the dish. Suction from release of the bulb removed mucous and adhering bits of tissue and sperm. Treated in this way, eggs were much less prone to attack by fungus.

Fertilized eggs and offspring were reared in dechlorinated freshwater at the Vancouver Public Aquarium. The eggs were transferred to cup-shaped rafts of celluloid mesh that floated on the surface in jars holding 500 cc of water. Each jar was vigorously aerated causing the raft of eggs to slowly rotate just beneath the surface. Eggs were incubated at a constant temperature of either 14 C or 18 C, controlled with thermostats suspended in water baths, and under a daily photoperiod of 16 hr. If fungus began to develop on eggs, they were treated with a 1:200,000 dilution of Malachite Green solution for 1 hr. One day after hatching, the fry were transferred to and reared in 8- or 10-gal

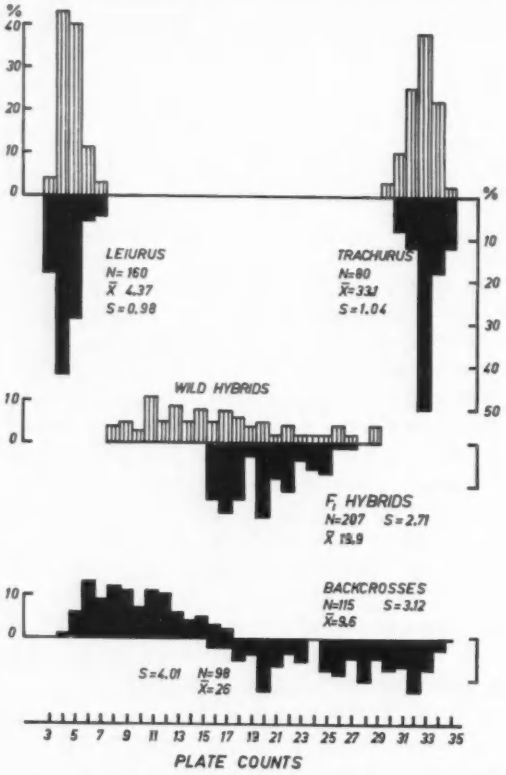

FIG. 10. Plate counts of laboratory-reared sticklebacks (black histograms) and of wild caught fish (hatched histograms). *Leiurus* used as parents to obtain offspring had plate counts from 3 to 5; *trachurus* counts were 32 to 34. Data based on 6 *leiurus* (intraspecific) crosses, 6 *trachurus* (intraspecific) crosses, 7 F_1 hybrid crosses (interspecific), and 7 backcrosses to *leiurus* and to *trachurus*. Approximately equal numbers of reciprocal crosses made.

aquaria kept at room temperature (18 C to 22 C). Fry were fed infusoria as soon as the yolk sac was absorbed, and at 4 days they began to feed on nauplii of live brine shrimp. After 8 weeks they were fed frozen brine shrimp until they were preserved for morphological analysis at 18 weeks. At that age they were between 2.8 and 3.7 cm long.

Lateral Plates.— It is clear from the results obtained with laboratory-reared fish that numbers of plates are genetically controlled (Fig. 10). Although the fish were reared uniformly, the numbers of plates in *leiurus* and in *trachurus* progeny are separated by a wide gap. Furthermore, the range of variation for plates of laboratory-reared fish is the same as that for wild-caught fish, except that no laboratory-reared *trachurus* had counts as low as 30 (nor did their

parents). The F_1 hybrids are largely intermediate. But a slight shift towards *trachurus* suggests some dominance by that form. The range of variation expressed in F_1 hybrids accounts for only a part of the variability encountered in wild hybrids, which also confirms the presence of backcrossing in the hybrid zone.

Backcross progeny obtained from crossing putative F_1 hybrids with *leiurus* have plate counts from 4 to 17. Of the total, 66% of the offspring are intermediate in counts between F_1's and *leiurus*, and can, therefore, be recognized as backcrosses using plate counts alone. But 29% of the backcrosses have counts from 4 to 7, and they would be identified as *leiurus* if plates were used as the sole criterion for identification. Of the offspring obtained from backcrosses to *trachurus* only 16% can be recognized as such; 32% have counts like those of *trachurus* and the remaining progeny (52%) have counts that overlap with F_1 hybrids. Backcrossing, then, accounts for the total spectrum of variation in plate counts between the two forms.

Gill Rakers.— Results from raker counts (Fig. 11) demonstrate that the divergence in gill-raker numbers between *leiurus* and *trachurus* is also under genetic control. The variations and modes for counts of offspring of *leiurus* and trachurus are like those for the wild populations. F_1 hybrids are intermediate

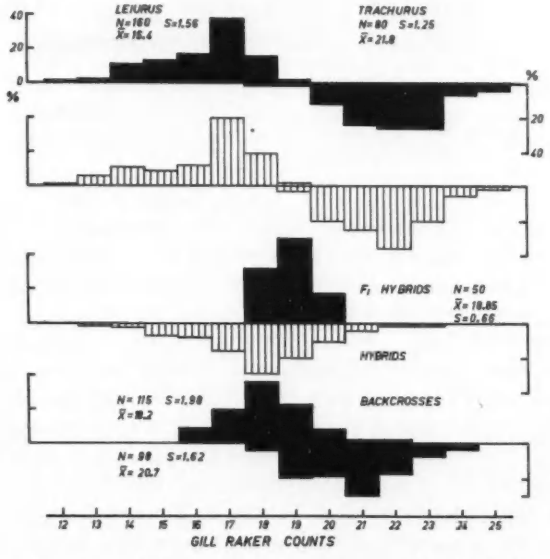

FIG. 11. Gill raker counts for laboratory-reared sticklebacks (black histograms) and for wild caught fish (hatched histograms). Data based on 6 *leiurus* (intraspecific) crosses, 6 *trachurus* (intraspecific) crosses, 7 F_1 hybrid crosses (interspecific), and 7 backcrosses to *leiurus* and to *trachurus*. Approximately equal numbers of reciprocal crosses made.

between the forms and are less variable than wild hybrids, so that these counts, like plate counts, confirm the presence of backcrosses in the hybrid zone. With backcrossing, variation is increased, and the modes for raker counts are intermediate between F_1's and the respective parental form. Although the range of variation in laboratory-reared hybrids is less than in wild hybrids, repeated backcrossing could produce the variation found in the wild. Counts of rakers and plates of the progeny reinforce the conclusions drawn from the hybrid index, that in the hybrid zone F_1's are relatively few and backcrosses to *trachurus* are rare. For if F_1's were present in the wild they could be recognized by either plate or raker counts. Also, the data reinforce the conclusion that most of the wild hybrids are backcrosses to *leiurus*.

Body Depth into Standard Length.— The difference in body shape between *leiurus* and *trachurus* is under genetic control (Fig. 12), but in contrast to

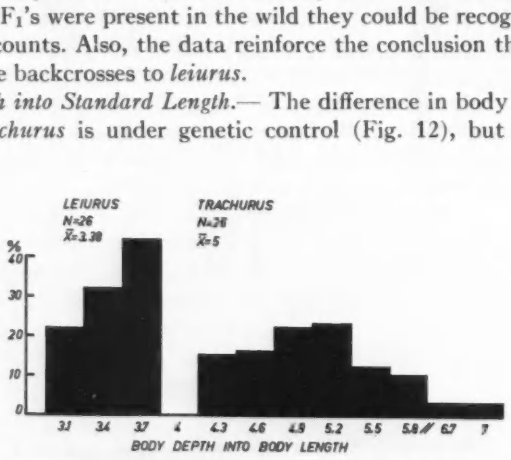

FIG. 12. Length–depth relationships for laboratory-reared fish. Data based on 6 parental crosses with *leiurus* and 6 parental crosses with *trachurus*.

wild fish, there is a noticeable gap between the laboratory-reared offspring of the two forms. This discrepancy might be caused by non-isometric growth so that adults of the two forms converge in this feature; or the greater variation in wild adults may be imposed by environmental variation.

Dorsal and Anal Rays.— Fin-ray counts for *leiurus*, *trachurus*, and hybrids are virtually alike when reared uniformly. *Leiurus* offspring (sample of 160) have dorsal ray counts ranging from 10 to 12 with a mean of 11.2 (s = 0.283); *trachurus* offspring (sample of 80), 10 to 13 with a mean of 11.4 (s = 0.803). For anal rays *leiurus* has a range of 7–9 and a mean of 7.35 (s = 0.048); *trachurus* 7–9 and a mean of 7.6 (s = 0.435). Hence, the difference encountered in the wild between the two forms must be due to environmental variation (phenotypic plasticity), not genetic divergence. It is for this reason that dorsal and anal rays were excluded from the hybrid index. The results make it plain that differences between races (or species) together with intermediates cannot be accepted without question as evidence for hybridization.

Other differences observed between the forms, though they were not quantified, include colour and shape of spines. Laboratory reared *leiurus* retain

their greyish-olive colour mottled with bands of brown or black, and their short spines, whereas *trachurus* remain a bright silver with long spines. Hybrids are variously intermediate. Genetic control of these features is thereby indicated.

SEASONAL ISOLATION

Materials and Methods

Breeding data were gathered over 2 years to determine what contribution differences in breeding season between *leiurus* and *trachurus* made to reproductive isolation. The study was largely confined to the Little Campbell River and local ponds, but observations were made in streams on Vancouver Island in the summer of 1965. Throughout the breeding season populations were censused at least twice weekly. *Trachurus* was sampled at Station N and *leiurus* at Station D, and because the heavily weighted lead line destroyed aquatic vegetation and presumably stickleback nests, sites for seining were rotated within a 1-mile section of stream at each station. Numbers of breeding fish were estimated from standard 15-ft seine hauls. Usually several hauls were made and the average catch for the series was calculated.

Breeding males were identified by their bright nuptial colours, and gravid females by their enormously swollen abdomens. The fine mesh of the seine captured recently hatched fry so that their earliest appearance could be recorded. It is assumed that nuptially coloured males and gravid females are breeding fish though this may not always be a reliable measure. Since gravid females were found later in the spring than nuptially coloured males and became nonreproductive earlier in the summer, it seemed most reasonable to record the beginning and end of the reproductive season by their presence and absence. The following criteria were used to indicate different phases of the breeding season: (*1*) Beginning of reproduction: estimated from the first gravid females collected in the season; (*2*) Occurrence of reproduction: estimated from the first fry collected in the season; (*3*) Peak of breeding: indicated by that period when the largest number of nuptially coloured males and gravid females were captured; (*4*) End of breeding season: indicated by the last gravid females collected in the season.

Little Campbell River

Leiurus and *trachurus* each have breeding seasons that last for about 4 months, but *leiurus* begins to breed much earlier in the year, starting in late February or early March (Fig. 13). The two forms breed at the same time for slightly more than 1 month (not counting early spawning *trachurus* which are very rare). The opportunity for interbreeding is further restricted, for when maximum numbers of one form are breeding, minimum numbers of the other are breeding. During the peak of the breeding season for *leiurus* there are very low numbers (<0.2 per standard seine haul) of early breeding *trachurus* present in the stream. Conversely, during the peak of the breeding season of *trachurus*, numbers of breeding *leiurus* have sharply declined.

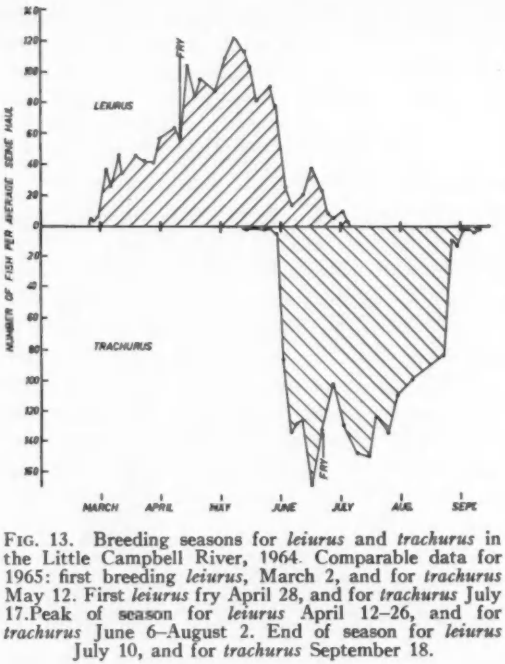

FIG. 13. Breeding seasons for *leiurus* and *trachurus* in the Little Campbell River, 1964. Comparable data for 1965: first breeding *leiurus*, March 2, and for *trachurus* May 12. First *leiurus* fry April 28, and for *trachurus* July 17. Peak of season for *leiurus* April 12–26, and for *trachurus* June 6–August 2. End of season for *leiurus* July 10, and for *trachurus* September 18.

Data for the breeding season of *leiurus* in ponds (D and M) resemble those from the stream but for one difference. In both ponds *leiurus* continues to breed later into the summer (in 1964 until August 5; in 1965 until July 16).

In passing, it may be mentioned that post-reproductive mortality was very noticeable in *leiurus*, beginning in late June and continuing throughout July. Their corpses were especially conspicuous in ponds. Many adults that were scarcely alive had either fungused bodies or eruptions on the caudal peduncle.

Early spawning *trachurus* make the chief contribution to hybridization in the Little Campbell River, and were collected in the stream both years. They preceded the principal breeding congregations into the stream by as much as 1 month (Fig. 13). They were further characterized by migrating farther upstream than individuals in the principal spawning migration, bypassing the *trachurus* breeding grounds (Station N), and thus establishing themselves in the hybrid zone (Fig. 7). Early migrants were collected at stations L, M, and M′ in mid-May. At the end of May, when the principal spawning migrations arrived in the stream, they settled at Station N, and there was no noticeable increase of *trachurus* in the hybrid zone at that time or later.

In May of 1965 I attempted to confirm these observations. During the first week in May a half-inch monofilament gill net was suspended across the

stream between the *trachurus* breeding grounds and the hybrid zone. Each day the net was searched for fish and a total of nine *trachurus* were captured over 6 days. The procedure was repeated the 2nd week in June, after the principal breeding migration of *trachurus* had arrived at Station N. None was captured.

The following data for catches of early and principal spawning migrants were gathered. In 1964, 26 *trachurus* (early migrants) were captured during the first 3 weeks of May within the hybrid zone; four individuals were taken at Station N. During the first 3 weeks of June, 19 *trachurus* were captured in the hybrid zone and thousands were present at Station N (see Table II). In 1965, 13 early migrant *trachurus* were captured during the first 3 weeks of May within the hybrid zone; three were taken at Station N. During the first 3 weeks of June, 14 *trachurus* were captured in the hybrid zone and hundreds were present at Station N (recall that a drastic reduction in density of *trachurus* occurred in 1965).

A comparison was made between plate counts of individuals from the principal breeding migration (sample of 404) and individuals from the early breeding migration (sample of 34). The ranges, means, and variances are the following: for the principal breeding migration, 30 to 35, $\overline{X} = 32.7$, $s^2 = 0.993$; for the early breeding migration, 33 to 36, $\overline{X} = 34.55$, $s^2 = 1.76$. Variances could not be assumed equal so that the approximation of *t*-test was used (Bailey, 1959). A value of $P = .001$ was obtained, verifying the observation that plate counts of early breeders average higher counts than those from the principal migration.

To recapitulate, early breeding migrants enter the stream about 1 month before the principal *trachurus* spawning migration, move farther upstream and settle in the hybrid zone at sites where hybrid swarms have developed (stations M and M′), and have significantly higher plate counts. All these facts point to the conclusion that these individuals are genetically different from individuals within the principal breeding migration.

Numerous nuptially coloured male and gravid female hybrids were collected in the hybrid zone and in Pond M. A most unusual relationship was found (Table III). The results from both years demonstrate an obvious seasonal isolation between the sexes of hybrids. Breeding males were taken in April and May and gravid females in June and July. In effect, then, the breeding season of hybrid males overlaps with that of *leiurus*, and the breeding season of hybrid females overlaps with that of *trachurus*. This physiological trait seems to be inherited as a sex-limited character such that breeding season of *leiurus* is dominant in hybrid males and breeding season of *trachurus* is dominant in hybrid females. Selander (1964) also reported sex-limited characters in hybrid birds.

Pond M is small, about $\frac{3}{4}$ acre, with extensive shallows and dense emergent vegetation. Sticklebacks were always collected by seining completely around the shore, and since seining in the stream is also efficient, it seems unlikely that results are spurious.

TABLE III. Numbers of breeding hybrids taken on dates indicated.

1964			1965		
Date	♂	♀	Date	♂	♀
April 6	9	0	April 2	17	0
10	23	0	8	21	0
12	13	0	14	20	0
18	18	0	20	9	0
25	41	0	24	8	0
May 3	14	0	29	23	0
5	21	0	May 8	15	0
13	27	0	16	16	0
27	11	0	30	6	7
June 3	0	0	June 3	5	12
7	0	14	12	0	24
9	0	26	14	0	27
15	0	28	24	0	16
18	0	18	28	0	19
26	0	15			
29	0	22			
July 5	0	10			

VANCOUVER ISLAND POPULATIONS

Beginning June 15, 1965, an 8-day field trip was made to various streams on Vancouver Island to find out if seasonal isolation was more widespread. If so, *leiurus* should have completed the breeding season at that time, and *trachurus* should be at the peak of the breeding season. The four streams listed in Table IV were sampled at frequent intervals from the estuary to the headwaters, but no *leiurus* could be found in two of the streams. All populations of *trachurus* sampled were breeding, and as expected no breeding populations of *leiurus* were found. A landlocked population of *trachurus* existed in a small beaver marsh adjacent to French Creek, and its banks were carefully investigated to ensure that the population was confined. About 100 *trachurus* were seined and inspected; almost all were in breeding condition. This provides additional evidence (along with evidence from hybrids) that the difference in breeding season between *leiurus* and *trachurus* is under genetic control. For if the difference were triggered by environmental factors, *trachurus* trapped in freshwater should not have been in breeding condition, as *leiurus* in local freshwater ponds were not.

In the Big Qualicum River, *trachurus* were breeding near the estuary, and it is noteworthy that nine individuals were taken farther upstream (7 miles inland), just below Horne Lake. These individuals, like early breeders in the Little Campbell River, had unusually high plate counts with a range from 33 to 36 and a mean of 35.2.

ETHOLOGICAL ISOLATION

Specific courtship displays that provide for the choice of conspecific mates, and thus prevent hybridization, constitute an important category of isolating mechanisms in animals. When isolating mechanisms are at issue it is essential that mate preference be investigated. Reproductive behaviour of the threespine stickleback is well known, but nothing is known regarding its courtship behaviour as an isolating mechanism.

Materials and Methods

Methods described by van Iersel (1953) were efficient for holding sticklebacks prior to mate preference tests. Large numbers of males of both forms were held in a 200-gal cement tank, the two forms being separated with a wooden partition that divided the tank in half. A constant flow of dechlorinated tap water provided circulation and a nearly uniform temperature of 8 C while a controlled photoperiod gave 16 hr of light. The low water temperature and crowded conditions kept fish at a decreased level of sexual excitation and minimized fighting. Gravid females that were ready to mate were almost impossible to hold in the laboratory for more than a day because they would spontaneously drop their eggs. Hence, fresh females were collected at the Little Campbell River just preceding mate preference experiments.

Sixteen aquaria (47 cm × 29 cm), each holding 8 gal, were uniformly prepared for mate preference tests. The bottoms were covered with a layer of sand 5 cm deep, and a dense covering of the plant, *Riccia*, floated on the surface. Water temperature in the aquaria fluctuated between 17 and 19 C.

Sixteen of the most brightly coloured males were then selected, eight each of *leiurus* and *trachurus*, and transferred from the holding tank to the aquaria. The sudden increase in temperature and the shift from crowded to solitary conditions usually produced a rapid onset of reproductive activities. First, nests of *Riccia* are constructed in the sand. Once a nest is made, the male burrows through the ball of fibers leaving an entrance and an exit and this action, called "creeping through", may be used as an indicator signifying the transition from nest building activities to courtship activities (van Iersel, 1953; Sevenster, 1961). Only after this activity had been observed were females introduced for tests. In most instances males completed nests about 3 days after they were introduced into the aquaria.

Each pair of females was selected so that they were as nearly equal in size and gravidity as possible. Difference in size between females never varied more than 3 mm. Once a pair of females was chosen they were presented to a "tester" male kept specifically for this purpose. If is well known that a female ready to mate will respond to the courtship of a male by performing "head up" (facing the male and tilting her body at a 45° angle). Females to be used in experiments were placed in glass tubes filled with water and suspended in the aquarium of the "tester" male. If both females responded to the courting male with "head up" this was taken as a reliable indication that each female

was prepared to mate, and the experiment continued. If not, other females were tested until two responsive ones were found.

Two females, one each of *leiurus* and *trachurus*, that were of equal size, gravidity, and preparedness to mate were then concurrently introduced into the aquarium of the experimental male. Courtship activities would begin and eventually, usually within 15 min, one of the females would respond to the male, follow him to his nest, and deposit her eggs there. This result was recorded in the closed sequential test design of Cole (1962), experiments continuing until significance or nonsignificance was achieved. After results were recorded for a male and the pair of females, the fish were discarded and experiments began in another aquarium.

Female preference tests were also conducted to detect a preference by females for males of the same form. A series of three 40-gal aquaria (70 cm × 35 cm) were used as experimental tanks. A strip of sand 5 cm wide was placed at both ends of each aquarium, and the intervening space between the two strips of sand was covered with coarse gravel. The depth of sand and gravel was uniformly 5 cm. A double glass partition with a space of 11 cm between partitions was placed in the centre of the aquarium so that each glass plate was equidistant from the strips of sand. A uniform layer of *Riccia* covered the surface of the water.

Males of approximately equal size (±3 mm) were introduced into the aquarium, *leiurus* on one side of the central partitions and *trachurus* on the other side. The arrangement of substrates forced males to build nests in the sand — they never attempted to build in gravel — and males were left until both had built nests and until "creeping through" had been observed. A gravid female was then selected and introduced between the glass partitions. Sometimes the female swam rapidly to the bottom and was not noticed by either male but eventually she would begin to swim about, as females usually did when first introduced, and males began to court her. The situation did not arise in which one male courted and the other male did not. Once the experiments were under way, observations were made by sitting quietly 1 m from the aquarium. If after 15 min a female had not responded to a male's courtship with "head up," she was replaced with another. When a female responded to either a *leiurus* or a *trachurus* male with "head up" the result was recorded in Cole's closed sequential test design. Tests were rotated between the three aquaria and continued until significance or nonsignificance was obtained.

Male Choice

The outcome of the first closed sequential test series for *leiurus* and *trachurus* males was not significant (7 *leiurus* to 6 *trachurus* females and 7 *leiurus* to 9 *trachurus* females, respectively), indicating no preference by the males. However, Cole (1962) points out that it is possible to commit a Type II error with this test, accepting the null hypothesis when a slight preference exists. To minimize this error a series of closed sequential tests were repeated until four replicates had been performed. In each of these the null hypothesis was

accepted. The outcome from each set of tests was then pooled to provide a more powerful test, and the results of these pooled totals for *leiurus* and *trachurus* males (26 *leiurus* to 28 *trachurus* females and 31 *leiurus* to 32 *trachurus* females, respectively) preclude the necessity for statistical treatment for present purposes. It may be concluded that males of either form reproduce with females of both forms randomly.

Female Choice

Again, in these experiments the outcome of the first sequential test series for *leiurus* and *trachurus* females was not significant (7 *leiurus* to 8 *trachurus* males and 10 *leiurus* to 8 *trachurus* males, respectively). The tests were replicated once and the pooled totals for *leiurus* and *trachurus* females (13 *leiurus* to 15 *trachurus* males and 18 *leiurus* to 19 *trachurus* males, respectively) obviate the necessity for statistical treatment. It may be concluded that ethological isolating mechanisms on behalf of females do not exist, or if they do, they exist at such an exceedingly low level that they play an insignificant role in reproductive isolation.

Hybrid Reproductive Activities

No mate preference could be detected by males or females of either form. It therefore seemed unlikely that hybrids would demonstrate a preference, and such experiments did not seem worthwhile. However, hybrid inviability is well known in many animals, and it was possible that hybrids might not be able to perform courtship activities, build nests, or perform parental care properly. With this in mind, a series of tests were designed using presumed F_1 and backcross hybrids.

Eight males that were putative F_1's and five males that were presumed backcrosses to *leiurus* (see section on Morphological Analysis) were collected and placed in aquaria like those described for mate preference tests. In every instance such males successfully built nests, courted, and spawned with female *leiurus*. In all these activities hybrid males seemed normal when compared with males of either form. After a clutch of eggs had been deposited in a male's nest his activities were observed each day. Hybrids were observed fanning, nudging, and gluing the nest. The eggs had hatched at 6 days, and observations continued for 2 more days. Males returned fry that had strayed from the nest, attacked a probe introduced into the aquarium, and pulled the nest into a loose aggregate of fibers. In all these activities their behaviour seemed normal. In one case a hybrid had two separate clutches of eggs deposited in his nest, one deposited on one day, the other on the following day. After the first clutch had hatched, he was very busy fanning the remaining clutch of eggs and returning wandering fry to the nest. There was no suggestion of hybrid inviability or breakdown in courtship or reproductive activities.

I had the impression that hybrid males executed parental care more vigourously than did males of either form. This was especially noticeable during fanning and returning fry that had wandered from the nest.

Finally, six hybrid females, presumably backcrosses to *trachurus*, were collected and their reproductive activities were examined. These females were placed in aquaria containing *trachurus* males that had built nests, one female in each aquarium. Five of the hybrid females responded to the male's courtship with "head up," and when the male led, the female followed to the nest, entered, and deposited her eggs. One female did not spawn, but no significance is attached to this result because there were frequently cases in which females of both forms failed to spawn. There were no suggestions of hybrid inviability or breakdown in hybrid females.

ECOLOGICAL ISOLATION

The two forms of sticklebacks occur in very dissimilar environments outside the breeding season, *leiurus* in freshwater and *trachurus* in the sea. Collections from the Little Campbell River during the breeding season demonstrate that even when the two forms are sympatric in the same stream, segregation exists. This suggests that each form is associated with different aspects of the environment producing what seems to be a very potent isolating mechanism. A series of experiments was designed to test for ecological factors that might be important in maintaining the observed segregation.

DISPERSAL EXPERIMENTS

Dispersal experiments were carried out with large numbers of *leiurus* by marking, releasing, and recapturing individuals moved from one part of their habitat to another. Transfer experiments, on the other hand, were conducted with *leiurus* and *trachurus* by transferring individuals of each form from their own habitat to the habitat of the other. For both series of experiments, fish were marked by clipping the first dorsal spine at its base. This mark proved to be reliable and easy to identify because captured fish erect their spines, and the absence of the large first dorsal spine is conspicuous. Six weeks before experiments began, 20 fish had their spines clipped with heavy nail-clippers and were held in the laboratory, and 15 others were clipped and held in screen live-boxes in the stream. After 4 weeks, two of the fish held in the laboratory and none of those held in the stream died. No stickleback showed evidence of regenerating their spines even though those held in the laboratory had grown. Moreover, there is no evidence that spines are used in locomotion and their absence should not hinder mobility.

Dispersal experiments were performed at Station D, a typical *leiurus* habitat. There are several miles of stream with easy access, narrow width, and shallow water — ideal conditions for recapture experiments. For the same reasons, stations D and N were used for transplant experiments.

Several observations point in the same direction, suggesting that *leiurus* is a sedentary fish. First, a population located at Station E has about 50% of the individuals infected with a parasite, *Neascus*, producing black spots on the body. A duck farm by the stream is probably responsible for the heavy

infection since ducks may act as the definitive host. The habitat is ideal and large numbers of *leiurus* are present in the densely vegetated shallows, yet within 300 ft either up- or downstream there are dense populations of *leiurus*, practically none of which are parasitized. Second, the cline in plate numbers found in *leiurus* (Fig. 6) occupies only 7½ miles. So brief a cline also suggests little movement among populations along the stream. Last, hybrids are confined to an amazingly narrow zone in the stream. Other factors may be involved, but localization of hybrids to points of hybridization (coexistence of *leiurus* and *trachurus*) suggests low dispersal rates.

On March 19, 1965, 2000 *leiurus* were collected at Pond D adjacent to the stream and transported immediately to Station D, where the spines were clipped and fish were released into the stream. Seining for recovery of marked fish began 2 days after their introduction and was continuous up- and downstream from the point of release. The procedure was terminated after a series of six or more consecutive hauls yielded no marked fish. Virtually no *leiurus* were recaptured beyond 600 ft from the point of release (Fig. 14). Most were recovered within 100 ft up- or downstream, and this was so even 30 days later when the experiment was terminated. Six months later collections at Station D turned up large numbers of marked *leiurus*. As *leiurus* always do, marked fish demonstrated a strong tendency to gather in densely vegetated shallows along the stream, and peaks of density shown in Fig. 14 correspond with such locations.

Transfer Experiments.— These experiments were undertaken to seek an explanation to the observed segregation between forms. Experiments began March 6, 1965 (earlier than the previous experiments) when 3081 *leiurus* were collected at Station D, marked, and released at Station N just below the point where Highway 99 crosses the stream. At that time *trachurus* had not entered

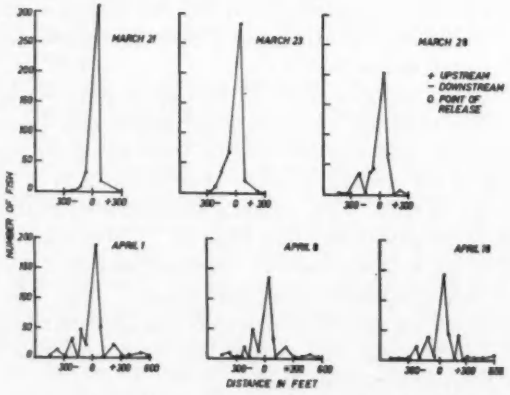

FIG. 14. Recapture data for *leiurus* transferred from Pond D to Station D on March 19. In the figure, zero represents point of release; + is upstream, − is downstream.

the stream. Recapture of marked fish began 6 days after their release, but not one *leiurus* was recovered although a distance of 1 mile up- and downstream was seined. Spot seining began at the estuary and continued upstream to Station N but none was found. This unexpected and sudden disappearance was probably a result of dispersal (predators were unknown in the area). Another transplant was attempted on March 14 using 6000 *leiurus*, and recapture began the following day. Very few individuals were recovered even the 1st day after release (Fig. 15), and after 3 days only three *leiurus* were taken over a 1- mile section of stream. The data suggest that most of the fish moved upstream

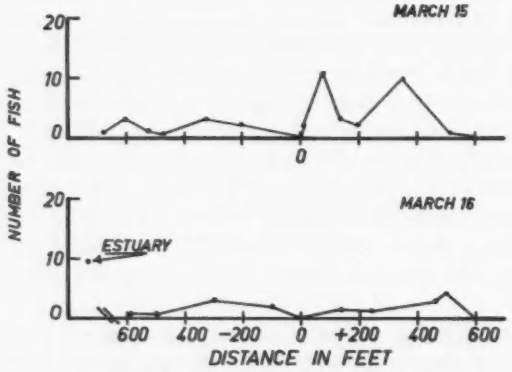

FIG. 15. Recapture data for the transfer of *leiurus* from Station D to Station N on March 14.

despite a current of 92 cm/second which they had to forge while passing through a 230-ft conduit beneath Highway 99. The results of these experiments contrast vividly with dispersal experiments in which *leiurus* was moved from one part of its habitat to another.

Several prominent changes occur at Station N between early spring and early summer. At the time the foregoing experiments were performed, the area is devoid of vegetation, and current is swift, there being no placid backwaters along the margins of the stream. In early June dense beds of *Elodea* cover the bottom and current is mild. This seasonal difference at Station N called for still another transplant experiment. On May 31, 3000 *leiurus* were marked and released. A population was successfully founded, and 24 days later when recapture stopped large numbers were still present (Fig. 16). Recovery for marked fish continued at monthly intervals and *leiurus* remained abundant until December 7 when none was caught. By that time the *Elodea* was not present, and the current was again fast.

The data from transfer experiments show several points in agreement with those obtained from transplant experiments with *leiurus*. First, there was a very noticeable shift upstream in the distribution pattern from the point of

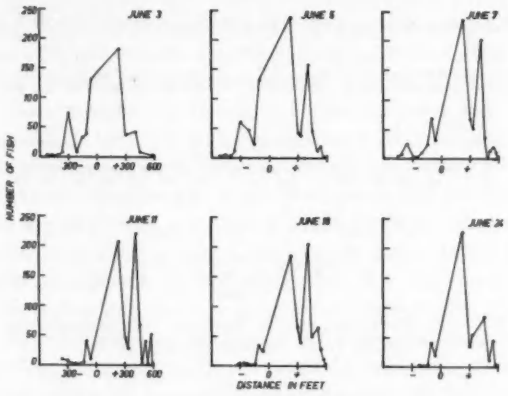

FIG. 16. Recapture data for *leiurus* transplanted from Station D to Station N.

release, which persisted throughout the summer. Secondly, *leiurus* proved to have a low dispersal, settling within 600 ft from the point of release and gravitating toward localities where vegetation was most dense and current least.

By June 2 *trachurus* were present at Station N, and the two forms coexisted for the rest of the summer (but recall that density of *trachurus* was very reduced in 1965). Extensive collections were not possible but those that were made suggest a marked increase in the incidence of hybridization as a result of the artificial introduction. This can be seen in the following collection data: September 18, 17 hybrids; October 12, 21 hybrids; December 7, 6 hybrids. Over the 2-year period prior to introduction, only 24 hybrids were collected at Station N. Despite the large numbers of *leiurus* that were introduced into the area, no pure *leiurus* offspring were recovered. Possibly the habitat is inappropriate in some respect, but aggressive interaction between males of the two forms cannot be ruled out.

Reciprocal transplants, moving *trachurus* from Station N to Station D, began on June 7, but marking the fish was not necessary inasmuch as *trachurus* were unknown in the upper reaches. Because *trachurus* were far less abundant in 1965, only 350 were collected and transplanted. Recapture began 2 days later. The characteristic upstream shift in distribution can again be seen (Fig. 17) but did not persist. *Trachurus* dispersed over a greater distance than *leirus* so that after 9 days they had moved at least 1000 ft downstream but just a short distance upstream. There was a continual reduction in the numbers caught and by June 27 few remained. A 1-mile stretch was again seined on July 19, 20, and 21 and none was captured, nor were any found while spot seining many sites in the upper reaches of the stream. The continual reduction in numbers of *trachurus* at Station D, together with little dispersal upstream and increasing numbers downstream, indicates a downstream migration from the area.

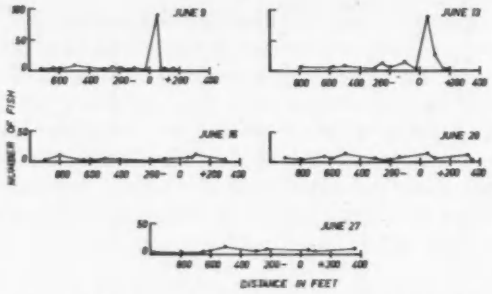

FIG. 17. Recapture data for *trachurus* transplanted from Station N to Station D on June 7.

During the 20 days that *trachurus* remained at Station D, they exhibited a strong preference for the few riffles that are present, where gravel and current prevail (most of the current is barely perceptible during the summer in the headwaters). Invariably the largest numbers were seined over such places giving rise to temporary clusters of *trachurus* that lodged in a very different portion of the habitat than did *leiurus*. It is unlikely that competition played a part in this habitat exclusion such that resident *leiurus* forced *trachurus* into those areas, for 14,000 *leiurus* had been culled from the area earlier in the spring. Active selection of these sites seems more likely.

Many of the *trachurus* that were caught seemed very unhealthy. Eleven dead fish were found, and males had lost their brilliant nuptial colours — which all introduced males had — whereas females assumed a dull silver colour rather than the bright silver that is typical. They were also emaciated. Mortality as well as dispersal from the area probably accounted for the continual reduction in numbers.

In summary, dispersal experiments confirm observations that *leiurus* is a very sedentary fish which congregates where vegetation is dense and current nil. When it is introduced into an environment devoid of vegetation with no retreat from current, it rapidly disperses from such an inhospitable habitat. *Trachurus* introduced into a *leiurus* habitat moves temporarily into localities of gravel and current, eventually disappearing from the area partly as a result of downstream migration and partly as a result of mortality; the inappropriateness of the habitat is demonstrated by their generally poor condition.

PREFERENCE TESTS

A series of preference tests were conducted in the laboratory in an attempt to define some of the factors in the environment that were important in securing ecological isolation. Four components seemed particularly important. First, *leiurus* was always observed nesting on a mud bottom and *trachurus* on a sand bottom. Secondly, *leiurus* nests were always found among broad-leaved vegetation and *trachurus* nests among *Elodea* and *Myriophyllum*. Thirdly, *leiurus*

dwells in standing water where nests are also found, whereas *trachurus* frequents and nests in gently flowing water. Fourthly, *leiurus* inhabits tea-coloured water and *trachurus* clear water. All four components may be important inasmuch as they may serve to separate the two forms into different habitats within the stream, thus providing for reproductive isolation (or competitive exclusion).

Substrate Preference.— In February, stocks of *trachurus* were collected from wharves in the sea near the estuary of the stream and held in the laboratory in 40-gal aquaria supplied with circulating seawater, a temperature of 8 C, and a photoperiod of 8 hr of light a day. *Leiurus* were taken from the stream, and held under similar conditions in freshwater. On April 1 the photoperiod was increased to 16 hr a day and the temperature was gradually elevated to 18 C. A dense layer of *Riccia* was supplied to all aquaria. These conditions promote sexual maturity (Baggerman, 1957). On April 15 *trachurus* were transferred to freshwater, and by May 28 many *leiurus* and *trachurus* had attained nuptial colours. Because stocks were held over glass-bottomed aquaria, there could not be any recent habituation to either a sand or a mud substrate.

Six 8-gal aquaria were prepared for substrate preference tests, mud from Station D and sand from Station N providing the materials. Aquaria were divided in half with a plate of glass; on one side a layer of mud was placed and on the other a layer of sand, all uniformly at a depth of 5 cm. The aquaria were then filled with water and after sediment had settled the partitions were removed and a layer of *Riccia* placed on the water surface.

Three nuptially coloured male *leiurus* and *trachurus* were removed from the stock tanks and introduced, one each, into the six prepared aquaria. They were left until they had built nests and were then replaced by other males. Each time a substrate was selected by a male of either form, the choice was entered in Cole's closed sequential test design and tests continued until significance or nonsignificance was achieved.

When sticklebacks build nests they dig a pit in the sand and later carry plant fibers to the pit gluing this together with a secretion, presumably from the kidney. It could be that secretions around the nest influence sites chosen by subsequent males, so the substrate around and beneath the nest was always removed after each experiment.

Results were highly significant, *leiurus* demonstrating a strong preference for nesting on mud (7:0), and *trachurus* a strong preference for nesting on sand (9:1).

Vegetation Preference.— The same basic design of aquaria and stocks of fish were used in these experiments as above. The bottoms of the aquaria were uniformly covered with sand and planted half with *Elodea* and half with *Oenanthe*, a headwater plant near which *leiurus* frequently nests. A space of 10 cm was left open on the observer's side and vegetation was planted in parallel rows 4 cm apart to facilitate observation.

Leiurus males showed a preference for nesting among *Oenanthe* (17–4) and *trachurus* males a preference for nesting among *Elodea* (9–1). Males twice built nests between rows of *Elodea* and *Oenanthe*, and the results were discarded.

The structure of the vegetation and its arrangement in the aquarium complicates drawing conclusions from the experiment. *Oenanthe* has long stalks free of leaves with a dense canopy of leaves above, but *Elodea* has small leaves that cluster densely along the entire length of the stalk. Thus, among *Oenanthe* the bottom is relatively open and there is a dense canopy 10–15 cm above. Among *Elodea* the cover above is less but the bottom of the tank is much more secluded. Rather than demonstrating a preference for type of vegetation, it is possible that the two forms demonstrated a preference for either a more open or a more secluded nesting site. Observations in the field, to be described, insinuate the latter possibility.

Current Preference.— An 8-gal aquarium was divided in half with a plate of frosted glass such that one end was firmly fitted against one side of the aquarium and the other end fell short of the opposite side of the aquarium by 12 cm. The bottom of the frosted plate was firmly wedged in 8 cm of sand. The top of the partition projected above the surface of the water so that the effects of a current generated on one side of the partition did not disturb water on the other side. The open end permitted free passage of a fish from one side to the other. A variable speed electrically driven motor with a 2.5-cm propeller was suspended just beneath the surface in a corner of the aquarium at the open end, and the device was held firm with a clamp and a ring stand. The propeller was directed so that a current was generated in one half of the aquarium but not in the other half. At the blind ends of the aquarium, mesh baskets with open bottoms were suspended and filled with *Riccia*. Pebbles were planted on the bottom at the open end of the aquarium.

This provided a tank in which fish could freely choose to nest in either current or standing water. At the open end where a slight current was detected, pebbles prevented sticklebacks from building nests. Fine threads or pieces of brine shrimp sank directly to the bottom on one side of the partition, but on the other side such items flowed gently through the water. Males placed in the aquarium quickly learned to pass freely from side to side, and there was no indication that they avoided the propeller, which at any rate was aside in a corner. Before and during nesting *trachurus* spent a great deal of time riding the current just in front of the rotor.

Both forms demonstrated a strong preference for nesting in standing water (7:0 for each), but this conclusion does not preclude the importance of current in segregating the two. *Leiurus* very rarely moved into the half of the aquarium with current and then only for a brief time. Before nesting began, *trachurus* was most often in the half with current, and even after it had built its nest in standing water, its activities aside from nest building were largely spent in moving water. These observations will be considered later.

WATER QUALITY PREFERENCE

For these experiments tea-coloured water from Station D and clear water from Station N were transported to the laboratory. The apparatus and methods used were like those described by McInerney (1964) for salinity preference

tests. In his experiments one chamber was filled with a given concentration of salt water, and freshwater from the adjacent chamber was allowed to rise above a central partition forming a bridge of water. The differential densities of salt and freshwater maintained the two separate and the bridge of water provided free passage so that the fish could choose between salinities.

In my experiments it was necessary to determine how much mixing occurred when water of equal density was placed in both chambers. A series of tests were made by dyeing water in the plugged chamber with Methyl Blue and then flooding to form a bridge. A very small portion of the dye moved into the flooded chamber as a result of turbulence, but the two clearly remained separate.

Equal numbers of fish were placed in each chamber, one containing tea-coloured water and the other containing clear water. After flooding, the numbers in each chamber were recorded at 15-min intervals. Sticklebacks do not school during the breeding season, so that social aggregation probably did not bias the results.

Preliminary experiments with stocks of fish that had been kept in the laboratory in clear water revealed a strong preference by both forms for clear water. Fresh stocks of *leiurus* (Station D) and *trachurus* (Station N) were, therefore, collected and used in later experiments (Table V). Again each form, in both replicates, exhibited a strong preference for clear water (from Station N) which became evident at 45 min and persisted for the duration of the experiment.

Ecological Observations on Vancouver Island

Observations on the effectiveness of ecological isolation were extended to four small streams on Vancouver Island. The streams (Table IV) were thoroughly sampled from the headwaters to the estuaries with a seine.

Table IV. Proportion of each form breeding in Vancouver Island streams, 1965.

Stream and date	*Leiurus*	*Trachurus*
Big Qualicum River June 15	Sample size — hundreds	Sample size — 30
	Breeding fish — 0 (fry to 2.1 cm abundant in adjacent marshes)	Breeding fish — 26(87%)
French Creek June 17	None present	Sample size — 163
		Breeding fish — 126 (77%)
Bonsall Creek June 18	Sample size — 96	Sample size — 72
	Breeding fish — 3(3%)	Breeding fish — 72
Sooke River June 20	None present	Sample size — 86
		Breeding fish — 86

Estuaries of all four streams have biotic and gross physical characteristics very much like the estuary of the Little Campbell River, including a sandy bottom, mixohaline waters (recorded with hydrometer), dense beds of *Ulva*, and the same species of fish. Breeding populations of *trachurus* were present near the estuaries of all four streams.

TABLE V. Preference tests with stream water. Numbers of fish in each compartment (tea-coloured water from station D, clear water from station N) at indicated times after introduction to apparatus.

	Time (*minutes*)							
	15	30	45	60	75	90	105	120
Leiurus								
#1. 30 fish								
Tea-coloured water	11	12	10	3	2	3	1	2
Clear water	19	18	20	27	28	17	29	28
#2. 20 fish								
Tea-coloured water	7	4	1	1	3	7	4	3
Clear water	13	16	19	19	17	13	16	17
Trachurus								
#1. 30 fish								
Tea-coloured water	27	13	9	7	3	4	7	2
Clear water	3	17	21	23	27	26	23	28
#2. 30 fish								
Tea-coloured water	16	17	12	6	3	1	0	0
Clear water	14	13	18	24	27	29	30	30

French Creek, Big Qualicum, and Sooke River are rather swiftly flowing streams with rocky bottoms and little vegetation. Sticklebacks were never found where these features prevail. As mentioned before (see Seasonal Isolation) nine *trachurus* were seined in the headwaters of Big Qualicum, all breeding males. The habitat had a sandy substrate, a mild current, and some *Myriophyllum* where nests were found. Also mentioned earlier was the beaver pond near the headwaters of French Creek that held a landlocked population of *trachurus*. The habitat was atypical for that form. The impounded water was choked with broad-leaved vegetation such as *Nuphar*, *Oenanthe*, and *Potamogeton*, the bottom was mud, the water was dark tea-colour, and there was no current. A dried stream bed led into French Creek, testimony that the waters once fed the creek. At one time *trachurus* may have migrated each year to spawn in the site now occupied by the impounded waters, but were trapped there upon completion of the dam. From a sample of 46 individuals, three were found that had plate counts like those of hybrids (15, 16, and 27). That they

were hybrids is questionable. The swift waters of French Creek are an unfavourable habitat for *leiurus* and none was found throughout its length, nor were any found in ponds along the stream. Typically, where *leiurus* is present, it is an abundant fish, especially so in ponds. The region is remote and it is not likely that man had recently introduced *leiurus*. Although one cannot rule out the possibility that those individuals found with reduced plate numbers were hybrids, it seems improbable. Alternatively, they may have arisen is a result of recombination from *trachurus* parents.

Leiurus was plentiful only in habitats with mud bottom, dense aquatic vegetation, standing or very placid and tea-coloured water. Although they were absent from most streams, they were abundant in ponds and marshes. Bonsall Creek is a small stream about half the length of the Little Campbell River, and the distribution of the forms there is worthy of special attention. A large breeding congregation of *trachurus* was found 1½ miles from the estuary where there was a sandy bottom, a mild current, and beds of *Elodea* and *Myriophyllum*. Within 200 ft upstream, the habitat changed to mud bottom with dense stands of *Potamogeton pulcher*, *Oenanthe*, and *Campanula*. In that habitat *leiurus* alone was collected. The shift in habitats along with a complete switch in forms over so brief a distance was compelling evidence supporting ecological isolation. More forceful evidence yet came from the intervening section where hybrids were common. In this zone the edges of the stream were muddy, heavily vegetated, and without current, and *leiurus* was most common. In the centre of the stream was sand, *Elodea* and *Myriophyllum*, and current. *Trachurus* were captured there in large numbers. Hybrids were common throughout this section composed of intermingled habitats, but not one was found just upstream or downstream although large samples were taken. In preserved samples of 82 fish from the hybrid zone, 21% were hybrids, whereas in a sample of 224 *leiurus* taken 100 ft upstream and 183 *trachurus* taken downstream, none were hybrids.

Nesting Sites and Materials

Data on locations of nesting sites and the materials used to construct nests were gathered at Station D for *leiurus* and at Station N for *trachurus*. Clarity of the water and aggressive behaviour of males with nests made nests surprisingly easy to find. If one sits quietly on the banks of the stream and watches for males that chase other sticklebacks or fry of coho salmon, eventually, such males will return to their nests to fan or to carry on with nest fabrication. When a nest was found, it was removed and placed in a vial of isopropyl alcohol. Depth of water, substrate, current, temperature, nearby vegetation, and location in the stream were recorded. Nests were later placed under a binocular dissecting microscope and the nesting materials were noted.

The following results were obtained from 26 *leiurus* nests: nests were always placed on a mud bottom and usually situated so that no current could be detected in the vicinity; average temperature was 21 C with a range of 16–23 C; nests were usually close to the banks and occasionally within 6 cm of

the waterline, with an average depth of 24 cm and sometimes as shallow as 4 cm; they were most commonly in pockets or indentations along the banks near logs or sticks, and although they were invariably found in the open, they were always close to stands of *Oenanthe*, *Potamogeton*, *Nuphar*, or *Carex*.

Results from a sample of 25 *trachurus* nests were the following: always on a sand bottom and so placed that currents ranging from 3 to 6 cm/second passed over the site; temperature averaged 17 C with a range from 14 to 22 C; nests were usually near the centre of the stream in water with an average depth of 50 cm; they were always either within or on the downstream side of a dense patch of *Elodea*.

Nests of *trachurus* were slightly raised from the substrate and had more noticeable entrances than those of *leiurus* because of the contrasting background of lightly coloured sand. Materials used in the fabrication of nests were often decomposed and could be identified only to a very general category. I could find no differences whatsoever in the kinds of material used by the two forms, which consisted of plant fibers, grass roots (usually fresh), bits of leaves, stems, and twigs.

Leiurus nests were always found in the open where current was absent, but *trachurus* nests were sheltered within or on the downstream side of thickets of *Elodea*. It is possible, then, that the difference found between *leiurus* and *trachurus* in the vegetation preferences tests represents a preference for more open or more secluded nesting locations.

Trophic Adaptations

Stomachs of adults of both forms were examined for possible differences in feeding that might explain the ecological exclusion. A sample of 30 individuals of each form was collected in July. Stomachs that were empty or that had food which could not be identified were discarded, and others were selected whose contents could be identified.

Samples were taken on the same days at Station D for *leiurus* and Station N for *trachurus*. Contents of the stomach were placed in a single layer on a grid divided into 5-mm squares, and food items were sorted into common lots. The percentage of each item was estimated for every stomach, and figures obtained for all stomachs were then summed and divided by total number of stomachs investigated. This method adequately expresses the food habits of a population for a given time and place.

Stomach contents were divided into three categories: bottom dwelling organisms, floating or free swimming organisms, and "uncertain," those that may fall into either of the two previous categories. Results (Fig. 18) suggest that *leiurus* feeds to a large extent on bottom dwelling organisms (78%) and that *trachurus* feeds on floating or swimming organisms (40%). However, *trachurus* eats a large amount of Chironomidae, which must be classified as uncertain because they may be free swimming or attached.

The validity of a difference in feeding habits may be questioned since the two forms were sampled from different habitats — habitats that may be expected to accommodate different invertebrate faunas (Table I). *Palaemonetes*,

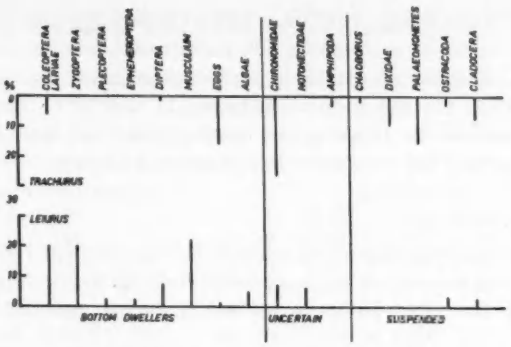

FIG. 18. Food items taken from stomachs of *leiurus* and *trachurus*.

for example, is common at Station N during June and July but is unknown from the upper reaches. However, *Musculium*, Zygoptera, and Coleoptera larvae are common at both stations yet they are important food items for *leiurus* only. Even when food items are commonly found in the two habitats one cannot be certain that they are equally available in both. However, the differences found imply a difference in feeding behaviour, one taking food largely from the bottom, and the other taking food suspended in the water.

POSTMATING ISOLATING MECHANISMS

Postmating isolating mechanisms include a host of factors that come into play after gametes have been shed and therefore do not prevent wastage of gametes as premating mechanisms do. The isolation springs from either genetic or chromosomal incompatibility between populations and may produce any of the following: gamete mortality, zygote mortality, hybrid inferiority, hybrid sterility, or hybrid breakdown. Any of these may serve to restrict gene flow between populations.

Methods for managing eggs and offspring were the following. Parents for crosses were taken from allopatric populations or from the hybrid zone, and eggs were usually fertilized on the same day that fish were collected. Eggs were incubated at a constant temperature of either 14 or 18 C. When the eggs hatched, the contents of the jar were transferred to an enamel tray so that the fry and unhatched eggs could be counted.

When hybrid crosses were made, the egg mass of each female was split into approximately equal halves. One half of the eggs was fertilized with homogametic sperm to serve as a control, and the other half was fertilized with heterogametic sperm and each hybrid cross was compared with its control. Thus, other causes of egg mortality were not attributed to genetic incompatibility.

Hybrid fry and controls were kept under uniform conditions, but because of limited facilities the different combinations of crosses extended over two

summers. The classes of offspring were held in separate gauze baskets 22 cm × 22 cm and 16 cm deep, with about 200 fish in each basket. All baskets were suspended in a 200-gal cement tank, and dechlorinated tap water circulated constantly, holding the temperature between 17 and 19 C. Experiments were terminated at 3½ months. Rearing and feeding procedure were like those given before for laboratory fish reared for morphological analysis.

Survival to Hatching

Comparing hatching success of reciprocal F_1 hybrids at both temperatures with their controls shows that the survival of hybrids to hatching approximates the survival of controls. There is no indication of genetic incompatibility (Table VI).

Table VI. Hatching success of hybrid crosses. Numbers in parentheses represent plate counts of parents. Controls presented in left-hand column and corresponding hybrid crosses in right-hand column.

	Cross	Hatched	Died	Mortality %		Cross	Hatched	Died	Mortality %
	Leiurus ♀ × *leiurus* ♂					*Leiurus* ♀ × *trachurus* ♂			
1.	♀(5) × ♂(4)	31	7	18	1.	♀(5) × ♂(31)	29	4	12
2.	♀(3) × ♂(5)	35	2	5	2.	♀(3) × ♂(33)	42	0	0
3.	♀(5) × ♂(5)	38	7	15	3.	♀(5) × ♂(34)	62	6	9
4.	♀(4) × ♂(5)	51	4	7	4.	♀(4) × ♂(33)	45	2	4
5.	♀(6) × ♂(5)	59	4	6	5.	♀(6) × ♂(32)	46	2	4
6.	♀(5) × ♂(6)	47	2	4	6.	♀(5) × ♂(34)	58	3	5
7.	♀(4) × ♂(5)	35	3	8	7.	♀(4) × ♂(32)	46	2	4
	Total			8.9		Total			5.8
	Leiurus ♀ × *leiurus* ♂					*Leiurus* ♀ × *trachurus* ♂			
1.	♀(4) × ♂(4)	57	1	2	1.	♀(4) × ♂(35)	33	1	3
2.	♀(4) × ♂(5)	50	2	4	2.	♀(4) × ♂(33)	39	3	7
3.	♀(5) × ♂(5)	31	7	18	3.	♀(5) × ♂(32)	33	4	11
4.	♀(5) × ♂(4)	41	1	2	4.	♀(5) × ♂(34)	35	3	8
5.	♀(6) × ♂(4)	43	4	8	5.	♀(6) × ♂(33)	29	3	9
6.	♀(4) × ♂(5)	28	3	10	6.	♀(4) × ♂(33)	46	10	18
7.	♀(5) × ♂(4)	54	2	3	7.	♀(5) × ♂(35)	53	1	2
	Total			6.1		Total			8.5
	Trachurus ♀ × *trachurus* ♂					*Trachurus* ♀ × *leiurus* ♂			
1.	♀(33) × ♂(32)	86	2	2	1.	♀(33) × ♂(5)	74	1	1
2.	♀(33) × ♂(33)	47	5	10	2.	♀(33) × ♂(5)	59	4	6
3.	♀(35) × ♂(32)	63	14	17	3.	♀(35) × ♂(6)	68	17	20
4.	♀(32) × ♂(33)	84	12	13	4.	♀(32) × ♂(5)	75	8	10
5.	♀(34) × ♂(35)	88	3	3	5.	♀(34) × ♂(4)	98	4	4
6.	♀(33) × ♂(31)	56	21	27	6.	♀(33) × ♂(5)	45	23	34
7.	♀(32) × ♂(33)	79	7	8	7.	♀(32) × ♂(6)	88	6	6
	Total			11.1		Total			11

(*Continued*)

TABLE VI. Hatching success of hybrid crosses. Numbers in parentheses represent plate counts of parents. Controls presented in left-hand column and corresponding hybrid crosses in right-hand column. — (*Concluded*)

	Cross	Hatched	Died	Mortality %		Cross	Hatched	Died	Mortality %
	Trachurus ♀ × *trachurus* ♂					*Trachurus* ♀ × *leiurus* ♂			
1.	♀(31) × ♂(35)	59	6	9	1.	♀(31) × ♂(5)	78	3	4
2.	♀(34) × ♂(33)	45	1	2	2.	♀(34) × ♂(6)	59	8	12
3.	♀(32) × ♂(35)	90	2	2	3.	♀(32) × ♂(5)	90	8	8
4.	♀(33) × ♂(33)	65	8	11	4.	♀(33) × ♂(4)	55	6	10
5.	♀(32) × ♂(33)	80	4	5	5.	♀(32) × ♂(5)	74	2	3
6.	♀(32) × ♂(31)	72	12	14	6.	♀(32) × ♂(5)	91	24	21
7.	♀(33) × ♂(32)	75	3	4	7.	♀(33) × ♂(6)	52	0	0
	Total			6.8		Total			9.3
	Leiurus ♀ × *leiurus* ♂			—		*Leiurus* ♀ × *hybrid* ♂			
1.	♀(6) × ♂(5)	18	4	18	1.	♀(6) × ♂(20)	56	2	3
2.	♀(5) × ♂(5)	31	0	0	2.	♀(5) × ♂(20)	39	3	7
3.	♀(4) × ♂(6)	25	2	7	3.	♀(4) × ♂(16)	66	1	1
4.	♀(4) × ♂(5)	32	1	3	4.	♀(4) × ♂(12)	48	0	0
5.	♀(5) × ♂(5)	34	2	5	5.	♀(5) × ♂(19)	33	1	3
6.	♀(4) × ♂(5)	19	0	0	6.	♀(4) × ♂(10)	49	3	6
7.	♀(4) × ♂(4)	39	5	11	7.	♀(4) × ♂(12)	43	4	8
8.	♀(4) × ♂(4)	72	6	8	8.	♀(18) × ♂(4)	62	2	3
	Total			6.8		Total			3.8
	Trachurus ♀ × *trachurus* ♂					*Trachurus* ♀ × *hybrid* ♂			
1.	♀(32) × ♂(33)	104	3	3	1.	♀(32) × ♂(21)	107	8	7
2.	♀(32) × ♂(34)	88	4	4	2.	♀(32) × ♂(11)	109	3	3
3.	♀(33) × ♂(32)	179	18	9	3.	♀(32) × ♂(16)	72	0	0
4.	♀(34) × ♂(33)	211	7	3	4.	♀(33) × ♂(15)	52	4	7
5.	♀(33) × ♂(33)	202	21	9	5.	♀(33) × ♂(16)	135	2	1
6.	♀(34) × ♂(33)	182	22	1	6.	♀(32) × ♂(14)	149	4	3
7.	♀(35) × ♂(33)	230	11	4	7.	♀(33) × ♂(20)	137	7	5
8.	♀(32) × ♂(34)	206	6	3	8.	♀(34) × ♂(18)	86	1	1
	Total			4.8		Total			3.3
	Hybrid ♀ × *leiurus* ♂					*Hybrid* ♀ × *hybrid* ♂			
1.	♀(20) × ♂(4)	44	0	0	1.	♀(20) × ♂(15)	62	5	7
2.	♀(19) × ♂(5)	53	3	5	2.	♀(19) × ♂(16)	67	7	9
3.	♀(15) × ♂(5)	36	1	3	3.	♀(15) × ♂(21)	81	2	2
	Total			2.9		Total			6.2

Backcrosses were made with hybrid males collected from the hybrid zone. During mid-May, backcrosses to *leiurus* were achieved and at the same time adequate hybrid males were taken and held in the laboratory to provide for backcrosses to *trachurus* in early June. When these backcrosses are compared with their controls, it may be seen that hatching success of backcrosses is like that for controls, and there is no suggestion of genetic incompatibility or hybrid breakdown (Table VI).

A restricted number of backcrosses to *leiurus* with hybrid females were also made as well as a limited number of F_2 crosses (seasonal isolation between sexes of hybrids made it difficult to collect mature individuals of both sexes at once). Controls were not provided, but hatching success within these groups compares favourably with survival of the homogametic crosses (Table VI). Again the results do not indicate genetic incompatibility or hybrid breakdown.

Survival of Fry

Mortality in F_1 hybrids was relatively high during the first 2 weeks after hatching, as it was in all classes of laboratory reared fish (Table VII). After 2

Table VII. Survival of laboratory-reared hybrids with controls presented in left-hand column.

Homogametic					Heterogametic				
	Mortality (%) at (*months*)					Mortality (%) at (*months*)			
Total hatchlings	½	1	3	4	Total hatchlings	½	1	3	4
1964									
	Leiurus × *leiurus*					*Leiurus* ♀ × *trachurus* ♂			
600	31	34	69	–	596	27	30	85	–
	Trachurus × *trachurus*					*Trachurus* ♀ × *leiurus* ♂			
994	32	36	79	–	1006	37	41	89	–
						F_1 Hybrid ♀ × *leiurus* ♂			
					122	92	100	–	–
1965									
	Leiurus × *leiurus*					*Leiurus* ♀ × F_1 *hybrid* ♂			
218	24	27	39	–	243	18	25	–	33
						Leiurus ♂ × F_1 *hybrid* ♀			
					61	23	29	–	36
						Trachurus ♂ × F_1 *hybrid* ♀			
					136	16	21	–	32
						Trachurus ♀ × F_1 *hybrid* ♂			
					74	22	30	–	34

weeks few died, but at 1 month a protozoan parasite, *Ichthyophthirius*, was introduced into the nursery from wild-caught fish. Fry were treated with standard saltwater remedy (Davis, 1961), which checked spread of the disease and cured many of the infected individuals. Mortality at the end of 3 months was comparable between F_1 hybrid classes and controls.

There was complete mortality in the backcross class to *leiurus*, which occurred at 33 days. It seemed improbable that disease or laboratory conditions

were responsible because all fish were held in a common supply of freely circulating water, and there was no comparable mortality in other groups of fry. At the time hybrid breakdown was suspected, so backcrosses to both *leiurus* and *trachurus* were made the following year.

Conditions for rearing fry were the same in 1965 except that at 2 months all fry were transferred from baskets to 40-gal aquaria provided with filters and aeration. In anticipation of the developmental mortality at 33 days, replicates of backcrosses were made at 2-week intervals so that, if death were a result of breakdown, there should be a 2-week interval among replicates when death occurred. The expected mortality did not eventuate (Table VII). Indeed, at the end of 4 months, survival of backcrosses was, if anything, slightly better than survival in the pure crosses. No explanation can be offered for the discrepancy in results, but it is important to recall that only one backcross combination was made in 1964 whereas all 4 were made in 1965, producing larger and more complete samples the 2nd year.

Despite good survival and seemingly normal and vigourous hybrid offspring, five F_1's and three backcrosses were abnormal. The deformities in all the abnormal hybrids were alike and consisted of the following: (*a*) no swim bladder; (*b*) intestine without the normal posterior loop, and consisting of a straight tube from mouth to anus; (*c*) abnormally large eyes; (*d*) dwarfed size, about half that of normal hybrids; (*e*) very thin and bow-shaped bodies. These individuals swam by fluttering the pectoral fins, and this along with an upset in their density resulting from the absence of a swim bladder produced a very erratic and jittery motion when swimming. They spent most of the time sheltered in *Riccia* on the surface, and if they were forced out they sank to the bottom and could return to the surface only with much effort. Hubbs (1927) reported similar abnormalities in parasitized fish, and since some eggs had been exposed to fungus at a late stage of development, it is not possible to attribute the abnormalities either to an incipent genetic incompatibility or to parasitism. If the cause were the former, it exists at an extremely low level.

At 4 months males had become aggressive and had blue irises suggesting they were approaching sexual maturity, but this ensued at a constant photoperiod of 16 hr and a relatively high temperature of 17–19 C (but see Baggerman, 1957). Gravid females were not found but neither was it possible to induce gravidity in partially mature females captured in the wild and kept in the laboratory.

Hybrid Fecundity

A measure of hybrid fecundity was derived from counts of mature eggs in hybrids as compared with counts from the two forms. Once it has been established that sterility barriers are not present, such counts of eggs can provide information about fecundity of hybrids, although they give no information concerning the number of clutches hybrids may produce.

The results (Table VIII) show that female hybrids are intermediate in fecundity between the two forms producing more eggs than *leiurus* but less

TABLE VIII. Numbers of mature eggs for hybrids and for each form (40 fish in each sample).

Leiurus				*Hybrids*			*Tracharus*		
Length	No. eggs	Length	No. eggs	Length	No. eggs	Length	No. eggs	Length	No. eggs
(*cm*)		(*cm*)		(*cm*)		(*cm*)		(*cm*)	
4.8	112	4.7	96	5.4	143	5.8	217	5.4	210	5.6	225
4.9	115	3.9	83	4.7	106	6	229	5.2	157	5.8	257
5.2	148	5	123	4.7	142	5.9	284	5.8	227	6.1	231
5.3	120	5.2	205	5.1	207	5.8	236	6	239	6.2	284
4	72	5.2	170	6	236	5.9	249	5.9	286	5.7	226
3.6	75	5	172			5.5	186	5.9	247	6	223
4.4	101	4.4	124	6.3	296	6	204	5.8	253	5.8	191
4	93	4.2	71	5.9	226	5.7	217	5.8	171	5.7	267
4.2	76	5.4	229	4.8	112	5.4	227	6	205	5.9	256
4	88	5.8	234	5.1	132	5.6	231	5.7	228	5.9	284
4.4	123	7.5	229	5.3	126	6.1	239	5.9	247	6	257
4.3	60	3.4	51	4.6	93	5.6	217	5.7	243	5.8	239
4.1	56	3.4	49	4	83	6	227	6.3	259	5.2	162
4.3	69	4.5	76	5.3	123	5.9	237	6.4	271	5.4	221
3.3	71	4.2	79	4.9	126	5.4	152	5.7	213	5.9	253
4.6	84	4.1	57	4.8	123	4.8	118	6	228	5.8	248
5.2	116	3.8	52	6	224	4.7	150	6.1	257	6.2	271
5.1	124	4.5	70	5.7	201	5.2	216	5.9	283	6.5	292
4.5	92	4	67	5.4	212	5.9	224	5.7	219	5.6	209
4.7	97	4.3	76	5.2	169	6.1	283	5.9	266	6	232
	$\bar{X}$ = 105				$\bar{X}$ = 190				$\bar{X}$ = 241		

than *trachurus*. This result is not surprising since egg production in each form is largely a function of size, and hybrids are intermediate between the two forms.

Hybrid Inferiority

It is exceedingly difficult to gain information pertaining to the selective disadvantage of hybrids relative to parental types, first because the selective agents operating against hybrids may be very low in intensity or very subtle in action, and second, because any evidence obtained in the laboratory for the absence of selection against hybrids is meaningless since the selective agents themselves may have been removed. In early September of 1964 the entire 1-mile hybrid zone was sampled and percentages of hybrids, *leiurus*, and *trachurus* estimated. The procedure was repeated again in early June of 1965, thus giving estimates of relative numbers of hybrids in autumn after reproduction ceased, and again in late spring. If hybrids were at a selective disadvantage relative to either form, one should be able to detect a decrease in proportion of hybrids over this 9-month lapse.

For September, census figures were: 217 *leiurus*, 14 *trachurus*, and 82 hybrids (26% of the sample); for June, they were 246 *leiurus*, 12 *trachurus*, and 73 hybrids (22% of the sample). This measure, rough though it is, gives little indication that hybrids suffer any manifest disadvantage as compared with parental types, and this together with an abundance of backcrosses in the hybrid zone indicates that hybrids are at little or no selective disadvantage in the hybrid zone.

Earlier when discussing migration, under the section on Seasonal Isolation, no mention was made of the behaviour of hybrids with respect to migration. Time did not permit an investigation of this important aspect, but the data given above for hybrids suggest that, whether they do or do not migrate, this factor does not constitute a severe selective disadvantage. For if so, a marked decline in numbers of hybrids should be found over the time interval concerned. However, the data are insufficient for any firm conclusions and the issue clearly needs more attention. Evidence given by Heuts (1947a) that hybrid offspring inherit maternal characteristics for salinity tolerance makes the need for further investigation plain.

DISCUSSION

Isolating Mechanisms

Morphological analysis and sampling in the field provided firm circumstantial evidence that *leiurus* and *trachurus* freely interbreed where they come together. In the Little Campbell River and Bonsall Creek the two forms are largely segregated, but where they cohabit, hybrids and backcrosses are plentiful. Furthermore, when *leiurus* was transplanted into a breeding population of *trachurus*, numerous hybrids arise. This evidence would seem to belittle the importance of behavioural or genetic obstacles to hybridization.

The virtual absence of behavioural and genetic barriers (but not of hybrid inferiority) to hybridization was confirmed by experimental analysis in the laboratory. Behavioural patterns that function as isolating mechanisms could not be detected, mating between the two forms being random in preference tests. In addition, hybrids successfully completed courtship and parental activities. Sterility barriers are apparently not present nor is genetic incompatibility, for even if the few abnormal hybrids found in the laboratory were a result of incompatibility (and not parasitism) it furnishes a very ineffective block to gene flow. Most of the hybrids were vigourous and no decrease in hatching success or survival of offspring could be found.

Seasonal isolation is partially developed. Both forms breed for a period of almost 4 months, but opportunity to interbreed is restricted to late May and June. When the principal spawning migration of *trachurus* enters the stream, the numbers of breeding *leiurus* are rapidly declining. In the Little Campbell River early spawning *trachurus* make the major contribution to hybridization. They arrive in the stream in mid-May during the peak of the *leiurus* breeding season, move farther upstream than does the principal spawning migration, and settle in an intermediate habitat where sparse but permanent populations of *leiurus* occur. Hybrid swarms have developed at these sites. This situation contrasts with that in Bonsall Creek where hybridization results from contiguous habitats and, therefore, contiguous populations of *leiurus* and *trachurus*. Several lines of evidence suggest that seasonal isolation has a strong genetic component. For example, the fact that landlocked *trachurus* were asynchronous in their breeding season with local populations of *leiurus*; that male and female hybrids inherit the breeding season of *leiurus* and *trachurus*, respectively; and that early spawning *trachurus* have significantly higher plate counts than those from the principal spawning migration; all point to the same conclusion.

Hybridization is greatly restricted by ecological isolation. *Leiurus* and *trachurus* are intimately associated with different habitats, and exclusion of habitats prevents biotic sympatry (Grant, 1963, makes a useful distinction between biotic and adjacent sympatry). *Leiurus* inhabits quiet and standing backwaters or ponds with a muddy bottom, dense stands of aquatic vegetation, and, usually, tea-coloured water. *Trachurus*, on the other hand, breeds in a habitat characterized by a sandy bottom and a mild curernt, beds of recumbent vegetation (*Elodea* or *Myriophyllum*), and clear water. These conditions held in all streams examined, but Bonsall Creek was especially instructive. In that stream a change in habitats occurs within 200 ft, and with it a complete break between the two forms. *Leiurus* is an abundant fish upstream in its typical habitat, and *trachurus* is numerous downstream in its habitat. In the brief intermediate zone, *trachurus* is present in midstream where there is sand, mild current, and *Elodea*, whereas along the margins of the stream *leiurus* occurs over a mud bottom, dense aquatic vegetation, and still water.

More evidence suggesting ecological isolation comes from dispersal and transfer experiments. When *leiurus* was moved from one portion of its habitat

to another it tended to sort out into quiet backwaters with dense vegetation along the margins of the stream. Large numbers of *leiurus* transferred in winter to the lower part of the stream (the summer breeding location of *trachurus*) rapidly moved out of the area. At that time of the year there was no retreat from current, and vegetation was not present. Such a hostile environment would prohibit *leiurus* from establishing permanent populations in the lower reaches of the stream, and thus prevent interbreeding. However, when *leiurus* was transferred to the same locality in the summer, it established residence where the current was nil and *Elodea* was dense. With the return of winter conditions, *leiurus* vanished from the area.

Conversely, when *trachurus* was planted in a *leiurus* habitat its numbers diminished over 1 month until none remained. There is evidence suggesting a downstream migration from the locale, but the loss of bright breeding colours, the thin and emaciated appearance, and the many dead individuals found, all indicate that *trachurus* is ill-adapted to the habitat. For the month they were present in the upper reaches, they congregated over relatively swift riffles with gravel bottoms.

Results of preference tests in the laboratory substantiate evidence from the field for ecological isolation. *Leiurus* had a strong preference for nesting on a mud substrate, and *trachurus* selected sand. *Leiurus* demonstrated a preference for nesting among *Oenanthe* (an exposed location), and *trachurus* a preference for nesting among *Elodea* (secluded at the nest site). Even though both forms chose to nest in standing water, *trachurus* spent much more time in the half of the tank with current than did *leiurus*. Results of preference tests were not always exclusive between forms, for even though *leiurus* is associated with tea-coloured water in the wild, it showed a preference for clear water from Station N.

In addition, evidence from morphology and behaviour indicates that the two forms are thoroughly adapted to different habitats. The terete shape found in *trachurus* is a well-known adaptation to life in current, and the deeper shape of *leiurus* an adaptation to life in standing water (Hubbs, 1940). It was demonstrated that distinct trophic adaptations exist between the two forms, *trachurus* possessing many gill rakers with a long and fine architecture, and *leiurus* with few gill rakers that are short and thick. In accord with this an analysis of stomach contents suggests that *leiurus* feeds predominately on bottom dwelling organisms, and that *trachurus* feeds largely on planktonic or suspended organisms.

Furthermore, observations on feeding behaviour in 40-gal aquaria indicate that *leiurus* feeds mostly on larger particles of food that have settled to the bottom, and *trachurus* feeds largely on suspended particles. This was especially noticeable when fish were fed frozen brine shrimp. *Leiurus* was often observed in the stream carrying on what I assume were feeding activities, and these very characteristic. The fish would swim slowly along just above the bottom and eventually come to an abrupt stop with the body tilted at about a 45° angle, linger for a moment, and dart forward into the mud causing a small

cloud to rise. After backing away it could be seen making a mulling motion with the mouth. This activity, or others that seemed associated with feeding, was never discerned in *trachurus*. In both the morphological and behavioural aspects of trophic specialization, the two forms are well adapted to their habitats, one for gathering food from the bottom in quiet, muddy, and vegetated habitats, and the other for taking suspended organisms in either a stream or in the sea where *trachurus* leads a pelagic existence.

Other differences concerning location of nesting sites may well be adaptations to life in standing or flowing water, which would further function to isolate the forms ecologically. *Leiurus* in the wild and in aquaria during preference tests chose open nesting locations. In still water, where they dwell, there would be no selective pressure favouring a protection of nests and fry from current that may sweep fry away from their parent. But there may be selection favouring an exposed nesting site where a male in a dense population would have a better opportunity to encounter a female, or the female to locate a nesting male.

On the other hand, *trachurus* always nests where current prevails. But evidence from collection of nests in streams, and from preference tests in the laboratory, demonstrates that *trachurus* protects its nest from current by choosing sheltered sites within dense beds of *Elodea*. In that habitat selection may be the other way round, as it were. There may then be a selective premium for placing nests where they are protected from current that could sweep fry from their parent.

Trachurus is more shy and retiring than *leiurus*. It is more difficult to induce *trachurus* to breed in an aquarium, and it spends more time hidden from view; in the wild, *trachurus* is equally shy and its nests more difficult to locate. A slight movement from an observer on the bank of the stream would send *trachurus* darting into a bed of *Elodea*. This contrasts with *leiurus*, which is easily observed and less shy. Again these differences in behaviour may be adaptations to the different habitats frequented by each, *leiurus* in a habitat with an obscuring mud background usually with a canopy of vegetation above, *trachurus* in an exposed habitat with a light background of sand and only recumbent vegetation.

The divergence that has ensued between the two forms is a result of selection and adaptation in two contrasting environments, producing manifold effects that are expressed in the morphology, habitat preference, and behaviour. The outcome of this selection is ecological isolation, which has a considerable genetic basis. Most of the morphological differences that distinguish the forms are under control of polygenes (an F_2 is required for rigorous proof), and it may be expected that other differences, such as habitat preferences, behaviour, and seasonal isolation, also represent considerable genetic differentiation. It is then somewhat surprising that isolating mechanisms so important in other animals, ethological and genetic incompatibility, have not developed as an incidental product. For as Mayr (1963) makes clear, the genotype is a coadapted and integrated unit, and each isolating mechansim has an extensive genetic basis.

The Hybrid Zone

Hybrids constitute 21% of the sticklebacks in the hybrid zone, although they may be as abundant as 46% at some localities (Station M and M′). The proportions of *trachurus*, hybrids, and *leiurus* are 1:8:10; but as mentioned, backcrosses to *leiurus* predominate. The composition of the hybrid classes seems clear. *Trachurus* is scarce and so will perpetuate hybridization by outcrossing with the more abundant *leiurus*. Once F_1's are produced, backcrossing to *leiurus* is most probable because of its greater numbers. Moreover, *leiurus* is a permanent resident in the hybrid zone and this provides considerable opportunity for backcrossing before *trachurus* enters the stream. The seasonal isolation between sexes of hybrids diminishes the chances for a F_2 generation.

A very important aspect of the hybrid zone that is not at all clear is its narrowness. Why has swamping not occurred? Evidence has been presented that both behavioural and genetic blocks to interbreeding are absent, and one would therefore expect unimpeded gene flow and a consequent breakdown in the divergence between *leiurus* and *trachurus*. Furthermore, no selection against hybrids could be detected inside the hybrid zone. Nevertheless, one is forced to assume that there is intense selection against hybrids since the hybrid zone in the Little Campbell River, and especially in Bonsall Creek, is exceedingly narrow (200 ft). The reverse cline found in *leiurus* further indicates strong counterselection against genetic exchange between the two forms. Clearly the duration of the hybrid zones cannot be established, but they are repeated many times in different streams. This suggests that swamping is not usual, and that such zones are relatively stable.

Thus, strong selective pressures against hybrids are indicated, yet cannot be detected in the laboratory or in the field. The absence of hybrids from the habitat of either parental type suggests that hybrid inferiority exists outside the hybrid zone, but because no inferiority could be detected inside the zone, it seems that hybrid inferiority is intimately bound to the environment. Evidence from several lines of investigation may provide a partial explanation for the very narrow hybrid zones.

(*1*) Considerable evidence was given that *leiurus* is a sedentary fish. Such a sedentary habit would reduce interchange between hybrids and either form. Fryer (1959) has demonstrated that parental care in cichlids serves to restrict dispersal of offspring, which become habituated to their local surroundings as a result of late emancipation from parents. The intense parental care in sticklebacks may well function to reduce dispersal as it does in cichlids.

(*2*) *Leiurus* and *trachurus* have strong preferences for different habitats. The hybrid zone, however, has characteristics that are intermediate between the habitats of the parental forms, and the drastic reduction in density of both forms in the hybrid zone testifies that it is less desirable. Hybrids have attributes that are intermediate between the two forms. They very likely prefer and select the intermediate zone, avoiding the *leiurus* and *trachurus* habitats that are just up- and downstream. The nonrandom distribution found in the hybrid classes provides evidence supporting this explanation. It may

be pointed out here that the intermediate counts found in anal and dorsal rays of hybrids are probably significant as regards the intermediate nature of the hybrid zone. The difference between parental types in fin ray counts was demonstrated not to be genetic, but environmental. Such differences can be caused by environmental temperature during development (Lindsey, 1962), and the fact that hybrids have intermediate counts provides additional evidence that the average temperature characteristics in the hybrid zone during the breeding season are intermediate between those of the "pure" habitats.

(*3*) *Leiurus* and *trachurus* each is well adapted to a specific habitat, and is poorly adapted to the habitat of the other. This is apparent from results of transfer experiments, morphology of the food gathering apparatus, choice of food, body shape, feeding behaviour, and choice of nesting location. Hybrids with their intermediate characteristics are presumably not as well adapted outside the hybrid zone as either parental type is. For example, hybrids are intermediate in number and structure of gill rakers, and may well be intermediate in their feeding behaviour. Thus, they are probably poorly adapted to collect either bottom dwelling or planktonic organisms, both because of inappropriate behaviour and structure. With intermediate body shapes they are adapted to neither standing nor flowing water. Or it is possible that hybrids exhibit an upset in choice of a nesting site, placing nests in open sites in current where fry would be swept away from the parent. If hybrids express unbalance in several or many adaptive traits, however subtle, this would impose a severe selective disadvantage on them. Furthermore, there are dense populations of *leiurus* upstream and dense populations of *trachurus* downstream from the hybrid zone. It is reasonable to suppose that hybrids are at a considerable disadvantage on either side of the zone, where they are poorly adapted, and must compete with well-adapted populations in a saturated habitat. But in the hybrid zone the selective disadvantage may be the other way round. Hybrids may be better adapted to the intermediate habitat than either parental form, and competition is greatly reduced because of a drastic reduction in numbers of both parental forms.

The circumstances explained above, with selection against hybrids outside the hybrid zone but no selection against hybrids inside the zone, could give rise to a stable hybrid zone in which neither swamping nor reinforcement would take place for considerable time. Behavioural and genetic blocks have not accompanied divergence between *leiurus* and *trachurus* so that where they come into contact there is no means to prevent interbreeding. On the other hand, ecological isolation is well developed and contact is limited to contiguous habitats. Once contact is established, the extent of hybridization will depend upon the size of the intermediate zone, where hybrids are not at a selective disadvantage. If the intermediate zone is relatively broad, as it is in the Little Campbell River (1 mile), hybridization will be extensive, but if it is narrow as it is in Bonsall Creek (200 ft) hybridization will be severely restricted. In either instance the hybrid zone cannot broaden, nor can swamping take place, because of the selective disadvantage of hybrids outside the zone. Still hy-

bridization continues inside the hybrid zone because there is no isolating mechanism to prevent it, and because hybrids are not at a disadvantage.

The above explanation for narrow hybrid zones and for selective pressures acting against hybrids (which one is forced to assume) is based on inference. There is a critical need to investigate hybrid inferiority in order to clarify the situation encountered in the hybrid zone, for even though the selective disadvantages of hybrids must be large, they must also be subtle. Sibley (1961) has stressed that it is essential to determine whether selection is or is not against hybrids when one analyzes cases of hybridization. Yet in nearly every case involving hybrid zones, including the work of Sibley and Sibley (1964) on towhees, it has not been possible to ascertain the selective pressures against hybrids. One is always left to assume hybrids are at a selective disadvantage (Mayr, 1942; Mayr and Gilliard, 1952; Hubbs and Miller, 1943; Hubbs and Miller, 1953; Hubbs, 1959; Ingles and Biglione, 1952; Chapin, 1948; Remington, 1954; Sibley and Sibley, 1964; Dixon, 1955; and many more). How selection operates against hybrids and what the selective pressures are have yet to be demonstrated in any rigorous way.

Surely the prerequisite for understanding hybrid inferiority and hybrid zones is a thorough knowledge of adaptation in the parental types. Only after it is understood how each parental form is adapted to its habitat can one understand how hybrids are less well adapted, *in that habitat*. Furthermore, unless one has an intimate knowledge of adaptation in the wild, laboratory experiments may be of little assistance in resolving the action of selection, as in the case of sticklebacks. Even the detailed work of Heuts (1947a) concerning salinity tolerance in *Gasterosteus* is difficult to interpret in terms of selection. Although he could demonstrate that salinity tolerance of hybrid eggs was inherited, his experiments were performed at constant temperature or salinity. However, the environment is never constant, and temperatures changes measured in the field during the reproductive season were each day greater than the kinds of differences Heuts considered. His data show that a difference in temperature of 4 C (19 C and 23 C) completely alters the pattern of survival in relation to salinity. Yet, in the Little Campbell River, temperature may fluctuate 12 C in 24 hr.

Mayr (1963; p. 106) considers ecological isolation as a premating mechanism and hybrid inferiority as a postmating mechanism. Furthermore, he follows Mecham (1961), who criticized Dobzhansky's theory of the reinforcement of isolating mechanisms. Mayr (1963) states "There is a fundamental difference between the two types: premating mechanisms prevent wastage of gametes and so are highly susceptible to improvement by natural selection; postmating mechanisms do not prevent wastage of gametes, and their improvement by natural selection is difficult and indirect."

This stated difference in the mode of operation of natural selection for pre- and postmating mechanisms is, however, not always valid. In sticklebacks, ecological isolation (premating mechanism) and hybrid inferiority (postmating mechanism) are obviously one. The two cannot be distinguished. Each form

is well adapted to a different habitat and this adaptation (ecological isolation) produces hybrids which are poorly adapted (hybrid inferiority) because they are intermediate. The selection that produces ecological isolation is the same as that producing hybrid inferiority. There are numerous examples of hybrid zones where it seems that ecological isolation and hybrid inferiority are one (Mayr, 1942; Mayr and Gilliard, 1952; Hubbs and Miller, 1943; Hubbs, 1961; Hubbs, 1959; Ingles and Biglione, 1952; Chapin, 1948; Sibley and Sibley, 1964; Dixon, 1955; and others). It is doubtful, in all cases of hybrid zones, that ecological isolation and hybrid inferiority can be separated, and hence, whether a distinction can be made between the two modes of selection that Mayr indicates.

Specific Status of *leiurus* and *trachurus*

Mayr (1963) defines species as "Groups of actually or potentially interbreeding populations which are reproductively isolated from other such groups." Sibley (1961) and Bigelow (1965) clarified the definition with respect to instances where hybridization occurs, pointing out that one must attempt to determine whether or not hybrids are at a selective disadvantage relative to parental types. If hybrids are at a selective disadvantage, the specific status of the hybridizing populations is ensured — that is, gene flow will be more or less completely blocked between populations by natural selection. In time, reinforcement will perfect isolating mechanisms, and hybridization will diminish and eventually cease.

Isolating mechanisms have developed between *leiurus* and *trachurus;* the two are reproductively isolated, and there is considerable genetic divergence. There are numerous morphological differences between the two, and most of these differences have a polygenic basis. Other distinctions such as ecological isolation, seasonal isolation, physiological and behavioural attributes like anadromy, all exemplify a great deal of genetic divergence. Thus, populations of *leiurus* and *trachurus* in the Little Campbell River and on Vancouver Island fulfill the biological definition of species.

Isolating mechanisms have been discussed. It remains only to emphasize the efficiency with which natural selection prevents genetic exchange between the species. In the Little Campbell River, where hybridization is extensive, gene flow is blocked within 1 mile, and each species remains distinct on either side of this brief zone. There is no indication that introgression occurs. In fact, the reversed cline in *leiurus* demonstrates that intense selection counters genetic exchange. In Bonsall Creek hybridization is confined to a zone of 200 ft, also evidence of very stringent selection.

Since hybrids are at a selective disadvantage, one may expect to find evidence of reinforcing selection. According to the concept of reinforcement those individuals that have poorly developed isolating mechanisms will be most prone to hybridize, and will produce hybrids that are at a selective disadvantage. Individuals that have better- developed isolating mechanisms will be least prone to hybridize and will, therefore, leave more offspring. Thus, natural selection will perfect isolating mechanisms because genotypes that

hybridize will be lost from either species, and hybridization will be abolished. Moore (1957) has criticized this concept, pointing out that reinforcement is an *ad hoc* mechanism; that is, reinforcement of isolating mechanisms is only of selective value inside the hybrid zone because only within the zone are genotypes that hybridize at a selective disadvantage. Outside the hybrid zone such reinforced genotypes would be selectively neutral or even disadvantageous and could not spread beyond the zone.

The latter argument may apply to isolating mechanisms such as ethological isolation, preventing their spread beyond the hybrid zone. It seems unlikely that this criticism applies to ecological isolating mechanisms for the following reasons.

The process of adaptation in *leiurus* and *trachurus* to such diverse environments produces ecological isolation. Hence, ecological isolation develops *outside* the hybrid zone as a result of the continuous process of selection. Moreover, individuals of either species that have poorly developed ecological isolation will be more prone to move *into* the hybrid zone than individuals with well-developed ecological isolation. Once in the hybrid zone, the former are at a severe selective disadvantage. First, they are poorly adapted to the intermediate habitat, and secondly, they will probably hybridize. Genotypes producing hybrids will eventually be lost from both species because their progeny (hybrids) cannot get out of the hybrid zone (or if they do, they are severely selected against). In either instance, natural selection will reduce the frequency of such genotypes in both species. Thus, there is no concern to have reinforced isolating mechanisms spread beyond the hybrid zone. On the contrary, individuals with poorly developed ecological isolation move *into* the hybrid zone and are selected against. Individuals with better-developed isolating mechanisms will, therefore, increase in number, continually fewer individuals will move into the zone, and the result will be strict habitat selection. It is possible that the reverse cline in plate counts of *leiurus* is a result of such reinforcing selection for ecological isolation, especially so since plate counts seem to be closely bound with adaptation to habitat.

But no matter how well- developed ecological isolation becomes, it would always seem to be less than perfect. In sticklebacks ecological isolation might become so perfected as a result of reinforcement that strict habitat selection and adaptation would accrue. Even if this occurred, however, habitats may lie side by side as they do in Bonsall Creek, and straying of no more than a few feet may result in hybridization. Trautman (1948) reports that two species of fish (*Schilbeodes*) are normally well separated by ecological isolation, but habitats of each species may occasionally be within inches of one another, resulting in hybridization. Many similar examples are known in fish (Trautman, 1957; Hubbs, 1961; for reviews).

Farther afield, an examination of specimens of *leiurus* and *trachurus* from Alaska to Oregon (Museum, Institute of Fisheries, University of British Columbia) reveals that the morphological characteristics distinguishing the two

species in the Little Campbell River and on Vancouver Island also distinguish other populations. Investigation of the voluminous literature confirms the fact that the morphological and biological properties found in *leiurus* and *trachurus* in British Columbia are widespread, from Europe (Heuts, 1947a, b; Munzing, 1963) and Russia (Berg, 1965) to the Pacific. Evidently *leiurus* and *trachurus* remain distinct and reproductively isolated although occurring sympatrically over a vast sphere; they are "good" species. This is so despite the fact that a few instances of (what seem to be) hybrid swarms are known (Munzing, 1963). Clearly these landlocked "hybrid" populations are exceptions. As Svardson (1961) points out, the freshwater species should take the name *Gasterosteus aculeatus* since this was the one originally described by Linneaus. The marine species would then take the name *Gasterosteus trachurus*.

Evidence is accumulating that there are sibling species within the complex of *Gasterosteus*. *Gasterosteus wheatlandi* on the Atlantic coast is one such species (Hubbs, 1929; Scott and Crossman, 1964), and another possible species has recently been uncovered by J. D. McPhail on the coast of Washington (personal communication). A probable third species of very large size has recently been discovered by myself on the Queen Charlotte Islands, and is currently being investigated by E. E. Moodie. It seems that the genus is presently undergoing considerable differentation and speciation as a result of the last devastation wrought by glaciation.

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Preparation of Light-Salted Fish by Brining[1]

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ABSTRACT

Kench and pickle salting are being used for the preparation of both light- and heavy-salted cod (*Gadus morhua*). Because of the potential advantages of brine salting, especially mechanical adaptability, experiments were conducted to determine the practical merits of this process in the preparation of light-salted codfish of the Gaspé-cure type. In a batch operation using an equal weight of fish and brine, good results were obtained using 25% by weight salt solution and 5 hr of salting. With continuous circulation of brine of constant strength at 18 C, optimum conditions were achieved for salting using an 18% brine solution and an 8-hr exposure period. With a brine temperature of 10 C, at a brine concentration of 22%, a curing period of 12 hr was required. In each case the salted product must be "water horsed" for about 24 hr before drying.

The water content of the product obtained by brining was higher than that of conventionally prepared fish, but the drying rate was also higher resulting in an overall drying time that was nearly the same. With efficient drying, it was possible to obtain a product which resembled the regular Gaspé-cure in appearance and in chemical characteristics.

INTRODUCTION

For the preparation of salted codfish, a great variety of procedures have been developed depending upon climatic and geographical conditions, local customs, markets, and distances between fishing grounds and drying establishments. However, both light- and heavy-salted codfish are generally produced by kench or pickle salting (van Klaveran and Legendre, 1958, 1965).

The kench salting method is the oldest and also the simplest one. With this method layers of split fish are alternated with layers of salt and piled into stacks. More salt is applied to thick fish and to the more thick parts of normal-sized fish than to the thin fish and the thin parts of normal ones. The fish are stacked with the flesh side up, but the top layer is placed skin up. The resulting pickle which is formed from the water extract is allowed to drain off. For heavy-salted codfish, the method gives satisfactory results, but the products obtained contain an adhering salt layer which must be removed either by manual or mechanical means before drying. The finished product often lacks uniformity and, in addition, with light-salted fish, bacterial contamination occurs very easily because the product is continuously exposed to the atmosphere. Only when space is limited, for example on board ship, is this method still used.

In pickle salting, dry salt is applied to the fish as in kench salting, but the fish are put into tanks or tubs and the pickle formed is not allowed to drain

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away. For light-salted fish, the salt is distributed by hand only in the region where the backbone has been removed. Napes and tails are salted from the brine which is formed. With due precaution and experience, a uniform product can be obtained. In this process a separate wash is not required since the brine performs this function as the fish is removed from the tank. However, much labor is required as each fish has to be treated individually. This limits the possibilities of mechanization. Around the Baie des Chaleurs, namely the Gaspé peninsula and northern New Brunswick areas, climatic conditions are well suited to the preparation of very light-salted cod, and here the pickle salting method is currently used. The Gaspé-cure fish requires only 8–10 kg of salt per 100 kg of fresh (split) fish. The salt content of the fish at the end of curing is 4–6%, and these conditions are favourable for slime-forming bacteria. The fish are then dried to a water content of 36–38%. The amber-colored product, manufactured during the summer months, is exported mainly to Italy. Later in the year a modification of the process utilizes 12–15 kg of salt per 100 kg split fish and the resulting product is called Fall Cure. Consumers of this product demand a whiter appearance and a softer texture than that of Gaspé-cure. The product contains 45% water after drying. This is acceptable, since the product is sold mainly to the northern parts of the United States and this minimizes the possibility of spoilage during transport.

As outlined above, pickle salting is currently being used for the preparation of these light-salted codfish products. The method gives satisfactory results, but depends mainly on the experience and ability of the salter. It requires considerable manipulation since dry salt is being used, and each fish must be treated separately according to size and thickness. A later development that reduced handling was to salt the fish by direct immersion in a solution of salt and water. This method is known as brine salting and is not used extensively with codfish, most probably because of the danger of oversalting the thinner parts of the products.

In view of the potential advantages of brine salting and also because the method lends itself to mechanization, a series of experiments was carried out to determine the possible merits of the method for the preparation of light-salted codfish of the Gaspé-cure type.

EQUIPMENT

To minimize variations in the samples, only very fresh cod (landed immediately after the catch) were used. The fish were split by an experienced operator shortly after landing and immersed in brine.

For the brine-salting operation, two different sets of apparatus were used. In one series of experiments a tank was used in which an amount of split fish was immersed in an equal weight of brine of different strengths for varying lengths of time. This simple equipment was useful for obtaining preliminary data concerning strength of brine, length of operation, and quality of the final product. However, with this arrangement the strength of the brine decreased continuously during the procedure because of the penetration of salt into the

fish, and a new brine had to be prepared for each experiment. These experiments demonstrated that good results were possible with brine salting, and it was decided to construct a special apparatus which would maintain a continuous circulation of brine of constant strength through layers of split fish. The apparatus consisted of an aluminum tank in which an aluminum rack for supporting a series of holding trays could be placed. The trays consisted of an aluminum frame attached to nylon strings, and they were spaced 2 inches apart on the rack. The rack held eight trays with a total capacity of about 55 kg of split codfish. A pump attached to the tank provided adequate circulation of brine. With such an arrangement it is important that pipe connections to and from the circulating pump are situated below the brine level in the tank to avoid foaming. For the same reason, the agitation created by the circulating brine should not be too great. A control valve was therefore included in the system to provide flow regulation.

In a plant using brine salting, the above arrangement could be readily adapted for continuous operation. For example, by using a large tank holding several racks, one rack could be removed each time a new rack is placed in the tank. Obviously, in a continuous operation, the strength of the brine must be kept constant. This may be achieved by using density control to regulate the circulation of varying amounts of brine through a bed of solid salt.

With our equipment, saturated brine was added from time to time to maintain the required level of salt in the system. Because of the relatively large volume of brine compared with that of the fish, the variation in salt content was small (about 1.5%) for a single run and the concentration was easily adjusted after each experiment.

The drying process utilized an artificial dryer equipped with a scale to indicate and record continuously the weight loss. The dryer included a thermocouple control (Legendre, 1958) and the procedure followed was that described in a previous paper (Legendre, 1955) dealing with the artificial drying of Gaspé-cure fish.

The quality of the finished product was evaluated by the author and two competent government inspectors who were familiar with this type of fish. Taste evaluations were made by a group of people familiar with the particular taste of Gaspé-cure fish. In addition, some chemical characteristics were also determined (Cardin et al., 1962), and compared to those usually obtained with Gaspé-cure. This included measurements of the levels of trimethylamine and other volatiles.

EXPERIMENTS AND RESULTS

First, the relative merits of the three methods, namely Kench, pickle, and brine salting in the preparation of Gaspé-cure fish were compared. The procedures followed for kench and pickle salting have been described previously in this paper.

Brine Salting

BATCH OPERATION

For batch operation the procedure described above was followed. Brines of various strengths (12, 15, 20, and 25% salt by weight) were used and it was found that whereas 70 hr were required to complete the salting operation in a 12% solution, only 5 hr were necessary in a 25% salt solution. However, because of the very rapid bacterial action, it was practically impossible to dry successfully the fish that had been prepared in brines weaker than 25%.

During the salting operation, sodium chloride extracts water from the fish muscle. When using brine, however, the removal of water by the salt differs considerably from that achieved with dry salt (kench and pickle salting). With brine, the water content of the fish after salting was found to be much higher than with the dry salt methods. The stronger the brine, the higher the water content at the end of the salting operation. With a 25% salt solution the water content of fish was found practically unchanged probably because the time of immersion is much shorter with strong brines and the salt accumulates very rapidly in the surface layer before it penetrates deeper. This high concentration of salt near the surface also acts as a preservative and is very useful during the initial phase of the subsequent drying operation. Since these experiments were conducted in batches, the strength of the brine decreased gradually. With an initial 25% salt solution, the strength of the brine at the end of the operation was found to be 15%. In very strong brines, napes and tails may be oversalted since the quantity of fish in relation to the quantity of brine is rather critical for thin sections. In weaker brines, salting takes longer and drying becomes more difficult because of the possibilities of sliming. A continuous circulation of brine of constant strength should be the best way to use weaker brines.

The characteristic amber coloration of Gaspé-cure fish was more pronounced with brine salting than with the usual pickle-cure method. The results obtained with each of the three salting methods are shown in Table I. The greatest advantage of brine salting is the reduction in time and handling, which makes it readily adaptable to mechanization.

CONTINUOUS FLOW SYSTEM

Another series of experiments was conducted with an apparatus providing continuous circulation of brine of constant strength through layers of fish. The equipment is described above, and the initial experiments were conducted at about 18 C. Various concentrations ranging from 15 to 25% salt by weight were tried, and the most satisfactory products were obtained with an 18% brine solution and about 8 hr of salting. It was possible to obtain a good-quality product provided the drying was rapid and efficient. At 10 C comparable results were obtained with a brine concentration of 22% salt by weight and a curing period of 12 hr. At lower temperatures, the rate of salt penetration in fish decreases further. The brine concentration may be increased without danger

TABLE I. Comparison of various salting methods for the preparation of light-salted fish.

	Salting method		
	Kench	Pickle	Brine
Weight of split fish	100	100	100
Weight of salt (or brine)	11 (salt)	9 (salt)	100 of 25% brine
Length of operation	4 days	2 days	5 hr
Washing after salting	Yes	No	No
Weight loss (%)	18.5	16.5	2
Salt content %			
Dry basis	22.6	22.4	25.7
Wet basis	5.8	5.5	5.6
Water content % (after salting)	74.2	75.5	78.2
Appearance (after drying)	Uneven salt distribution	Good	Good; amber colour more pronounced
Remarks	Spoilage during salting	Fish must be treated individually	Suited to mechanization

of oversalting in order to reduce the time of the operation. (Optimal conditions are shown in Table II.)

The salted product was then "water horsed" and dried artificially up to the usual final moisture content of Gaspé-cure fish. After salting the fish are commonly laid on the floor for a few hours in order to permit removal of excess water from the surface of the product. The operation is called "water horsing." With brine salted fish, because of the high moisture content, it was found that "water horsing" for about 24 hr is essential. The operation aids in further penetration of salt into the product which equalizes the salt distribution and results in better appearance of the final product. The drying operation was then performed as rapidly as possible in order to avoid contamination on account of

TABLE II. Suitable conditions for the brine-salting process as used in the preparation of light-salted codfish of the Gaspé-cure type.

	Brine concentration (%)	Time (*hr*)	Temperature (*C*) (of brine)
Batch operation (equal weight of fish and brine)	25–15	5	18
Continuous circulation of brine of constant strength	18	8	18
	22	12	10

moisture. The moisture loss curve obtained with the artificial drying of brine-salted fish compared with that of conventional Gaspé-cure is shown in Fig. 1. Thermocouple control was used (Legendre, 1958) and three drying periods were necessary to complete the drying operation. Figure 1 shows the first

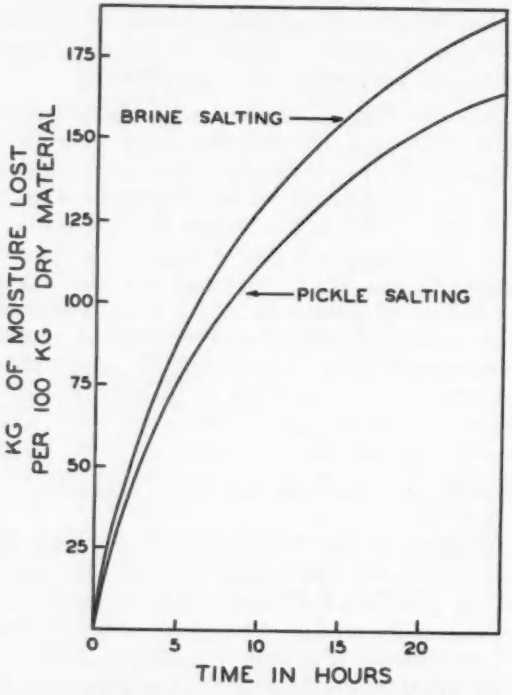

FIG. 1. Moisture loss curves obtained when drying artificially brine-salted codfish and conventional Gaspé-cure (pickle salting).

period only. As illustrated in the figure, moisture loss is greater and consequently the drying rate is faster with brine-salted fish. Since the moisture content of the brine-salted product was higher, the overall drying time was about the same as that of the conventional Gaspé-cure. With brine-salted fish, it is essential that the dryer has an adequate capacity to produce the required drying rate. Otherwise, the surface of the product will remain wet too long and encourage bacterial action, which will ultimately lead to a contaminated product.

The material obtained after brine salting and drying was of good appearance, practically indistinguishable from the conventional Gaspé-cure fish. However, it was thought possible that this rapid method of salting might result in producing a fish which looked like Gaspé-cure, but which would not

approach it in taste due to differences in the fermentation processes resulting from the changed conditions. It was therefore decided to assess the products of the pickling and brine salting processes by determination of volatile substances at various intervals during salting and drying. It was shown that with pickle salting the main production of volatiles takes place during salting and "water horsing." With brine salting the main production takes place during the drying operation. In brine-salted fish the overall content of volatile substances was somewhat lower than in fish prepared by the pickle salting method, but both products were in the general range of Gaspé-cure fish. It seems, therefore, that with the brine-salting method it is possible to obtain a product which not only looks like Gaspé-cure but also has the essential characteristics of the regular product.

ACKNOWLEDGMENTS

The technical assistance of Mr M. A. Bordeleau throughout the investigation and with special reference to the chemical determinations is gratefully acknowledged.

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Catecholamine and Carbohydrate Concentrations in Rainbow Trout (*Salmo gairdneri*) in Relation to Physical Disturbance[1]

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ABSTRACT

In rainbow trout (*Salmo gairdneri*) blood plasma concentrations of adrenaline (AD) and noradrenaline (NAD), and liver and heart concentrations of AD increased in response to severe physical disturbance. Skeletal muscle and anterior kidney concentrations of AD and NAD did not change detectably. Maximum plasma concentrations observed during disturbance of the fish were 0.20–0.36 μg AD and 0.05–0.09 μg NAD/ml. These plasma concentrations decreased relatively rapidly during recovery of the fish.

Plasma glucose concentrations increased in response to disturbance, the magnitude and duration of the hyperglycemia being greater in those fish with initially high liver glycogen reserves. Liver glycogen concentrations in those fish with initially high (ca. 7%) concentrations apparently decreased in response to disturbance and increased during recovery of the fish, but no change was detected in liver glycogen concentrations in fish in which they were initially low (ca. 2.5%). Heart and skeletal muscle glycogen concentrations decreased in response to disturbance and increased during recovery.

In skeletal muscle, the concentration of adenosine 3′,5′-phosphate and the proportion of phosphorylase in the *a* form increased in response to disturbance of the fish and decreased thereafter.

INTRODUCTION

THE OCCURRENCE of catecholamines in fish, their role in carbohydrate metabolism, and their effects on isolated, perfused fish hearts, fish gill vessels, intact circulation, stomach and alimentary canal, have been the object of a number of investigations (for references see Östlund, 1954; Barrington, 1957; Mott, 1957; Euler and Fänge, 1961; Plisetskaia, 1964). However, as far as we are aware, there are no published data regarding possible changes in the concentrations of catecholamines in the blood, skeletal muscle, or visceral organs of fish in response to stimulii that might be expected to bring about such changes. To obtain some knowledge of these possible changes, we attempted to measure the concentration of adrenaline (AD) and of noradrenaline (NAD) in blood, anterior kidney, liver, heart, and skeletal muscle of rainbow trout at rest, after short periods of severe physical disturbance, and at intervals thereafter during which the fish remained quiet. In addition, the corresponding concentrations of

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blood glucose and of liver, heart, and skeletal muscle glycogen were determined. A limited investigation was also made of accompanying changes in the concentration of adenosine 3′,5′- phosphate (cyclic AMP) and of the proportion of phosphorylase *a* in the skeletal muscle, both of which are known to be influenced in other animals by catecholamines (Sutherland and Rall, 1960).

METHODS AND MATERIALS

Rainbow trout, *Salmo gardneri*, (300–400 g in weight) were maintained at about 10 C on a diet consisting of a mixture of beef heart, beef liver, canned salmon, and pablum (in a weight ratio of 1:1:1:0.01). The aquarium tanks (20 × 42 × 16 inches in depth) were covered with black plastic sheets without completely excluding light. The size of the tanks made it possible to net and kill fish within a few seconds with a minimum of struggling.

Equal numbers of fish were placed in each of two or three tanks, were fed 7 days or longer, and were then starved for 2 days before the experimental treatment was begun. To obtain fish in a "rested" condition, only one fish was removed from each tank at the end of the 2-day starvation period and was killed as quickly as possible (for procedure see below). Fish were "disturbed" by repeatedly grasping them by the tail at short intervals thus forcing them to move vigorously about the tank. For recovery, the plastic cover was replaced on the tank and the fish left undisturbed and unfed. Periods of disturbance were varied between 2 and 120 min; periods of recovery between 1 and 48 hr. The fish appeared to be nearly exhausted after 15 min of disturbance. The procedure described was adopted after a number of preliminary experiments through which it was established that 2 days of starvation under our conditions of feeding and temperature resulted in a relatively low and reproducible level of glycemia, and that the disturbance and recovery periods were reasonably adequate for the purpose intended.

Fish were killed by thrusting a sharp knife through the spinal cord just posterior to the brain, after which they were bled by severing the caudal artery, the blood being collected in a small beaker containing anticoagulant solution (ethylenediaminetetraacetic acid (EDTA), 1% solution, *p*H 7.4). This process required about 1 min. The heart, liver, and lateral dorsal muscle, as desired, were then quickly removed and a small portion of each was macerated in 30% KOH solution for subsequent glycogen determination, the remainder being frozen in liquid nitrogen and stored on dry ice for use in catecholamine measurements. The anterior kidney was also removed, frozen in liquid nitrogen and stored on dry ice. This sampling and freezing procedure is slow in comparison with the procedure possible in work with perfused organs or with individual organs in situ in anaesthetized and operated animals (e.g., Drummond et al., 1964). Consequently, it is probable that the "resting" values for the various measurements made in the present work are not the true resting values, but for the kind of investigation we wished to conduct, there appeared to be no good alternative to the procedure adopted. For analyses, the blood, skeletal muscle, and organ samples of each group of fish (two or three fish) were

pooled. The pooling of blood and of organs was necessary to obtain a sample of sufficient size for catecholamine analysis. The values recorded in the tables and figures are therefore average values for such single groups, those recorded in Fig. 4 and Fig. 6–9 being for groups of three fish in all instances. Unfortunately, it was not possible to carry out sufficient determinations to permit statistical evaluation. However, the experiment described in Fig. 4 and Fig. 6–8 was repeated on three separate occasions and any obvious differences between the results of different experiments have been recorded in the text.

AD and NAD in blood plasma and in trichloroacetic acid extracts of skeletal muscle and organs were absorbed on alumina at *p*H 8.4, eluted with 0.3 M acetic acid solution, and then assayed by the trihydroxyindole-fluorimetric method (Crout, 1961; Euler and Fänge, 1961; Udenfriend, 1962). It was necessary to use EDTA in tissue extracts and in eluates from alumina in order to avoid serious interference in the determinations (Udenfriend, 1962). Glucose was determined by the glucose oxidase method ("Glucostat," Worthington Biochemical Corp.); glycogen by the concanavalin A method of Cifonelli and Smith (1955) with oyster glycogen (Eastman) as standard. Extraction of skeletal muscle glycogen phosphorylase was carried out essentially by the method of Posner et al. (1965). Phosphorylase *a* and total phosphorylase activities were assayed by the method of Danforth et al. (1962), phosphorylase *a* activity (measured in the absence of 5′-adenylic acid) being expressed as a percentage of the total phosphorylase ($a+b$) activity (measured in the presence of 5′-adenylic acid). Cyclic AMP was extracted from skeletal muscle by the method of Posner et al. (1965) and assayed according to the procedure of Posner et al. (1964), a crude phosphorylase *b* kinase solution being prepared in the same manner as glycogen phosphorylase (see above). The enzyme extract was diluted with four volumes of neutral 0.015 M cysteine solution just before use. Phosphorylase *b*, for use in the cyclic AMP assay, was prepared from skeletal muscle of unexercised trout (killed quickly, but not anaesthetized) by the method of Fischer and Krebs (1958), but omitting their crystallization step, which in our hands did not result in crystallization from the fish muscle extract, and substituting concentration by lyophilization. Such phosphorylase *b* preparations exhibited little or no phosphorylase *a* activity.

For paper chromatographic and fluorimetric identification of catecholamines from the blood and anterior kidney of trout, 60 fish were netted and killed directly from a fish farm pond and their blood and anterior kidneys were pooled (200 ml and 30 g, respectively). These fish were unavoidably exposed to disturbance during catching. The catecholamines were obtained by absorption on alumina (see above), and the eluates were concentrated by lyophilization and then subjected to descending chromatography on Whatman No. 1 paper with *n*-butanol saturated with N HCl as the developing solvent (Euler and Hamberg, 1949). The chromatograms were dried in air at room temperature, and the catecholamines were then located by spraying the paper with a 0.44% potassium ferricyanide solution in phosphate buffer, *p*H 7.8 (James, 1948).

For determination of excitation and fluorescence spectra of trihydroxyindole derivatives of compounds from trout blood and anterior kidney, unsprayed areas of paper chromatograms corresponding to the positions occupied by known AD and NAD markers were eluted with 0.5 M acetic acid solution in the dark at 0 C. The catecholamine content of the eluates was then determined by the trihydroxyindole procedure (see above) and diluted with deionized water to a suitable concentration for use in spectral measurements.

RESULTS

Chromatographic and fluorimetric evidence for the occurrence of AD and NAD in rainbow trout blood and anterior kidney is presented in Fig. 1, 2, and 3.

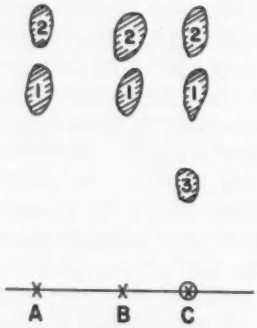

FIG. 1. Tracing of paper chromatogram of catecholamines: A, from rainbow trout blood; B, known reference compounds (1, noradrenaline; 2, adrenaline); C, from rainbow trout anterior kidney (spot number 3 unidentified, see text). Solvent: *n*-butanol saturated with N HCl. Paper: Whatman No. 1. Descending chromatography, 23 hr. Colour developed with potassium ferricyanide.

Chromatographic and biological evidence of the occurrence of both compounds has been given previously for chromaffin cells and visceral organs of several fish (e.g., Euler 1953; Shepherd and West, 1952 and 1953; Shepherd et al., 1953; Östlund, 1954; Augustinsson et al., 1956). In chromatograms of the trout anterior kidney, a third faint spot also giving a red–brown colour with potassium ferricyanide, and with an R_f about one-half that of NAD was present. The position of this spot appeared to correspond to that expected for "catechol-4" of Östlund (1954) but the compound was not investigated further.

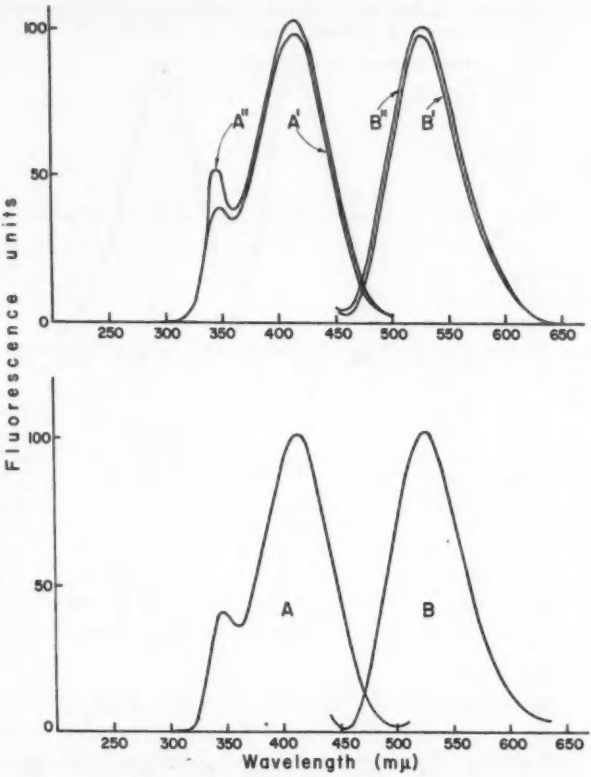

FIG. 2. Excitation (A) and fluorescence (B) spectra of adrenolutine prepared from known adrenaline (A and B), from trout blood (A′ and B′), and anterior kidney (A″ and B″). For preparation of excitation spectra, readings of fluorescence were made at 527 mμ; for fluorescence spectra, excitation was at 415 mμ.

Since it was thought the anterior kidney might be the principal source of catecholamines possibly entering the blood in response to disturbance of the fish, several experiments, in which the catecholamine content of blood plasma and anterior kidney was measured in trout at rest and after various periods of disturbance and recovery, were conducted. The results are presented in Tables I and II. In blood plasma, the concentrations of both AD and NAD increased in response to disturbance, that of AD to a greater extent. When disturbance of the fish was prolonged (Table I) the concentrations in blood plasma declined somewhat after about 15 min. During recovery after 10 min disturbance, the concentrations in blood plasma declined and returned to resting values within 24 hr (Table II). In the anterior kidney, which in *Salmo* is believed to contain cells possibly corresponding to the adrenal medulla of mammals (Overbeeke,

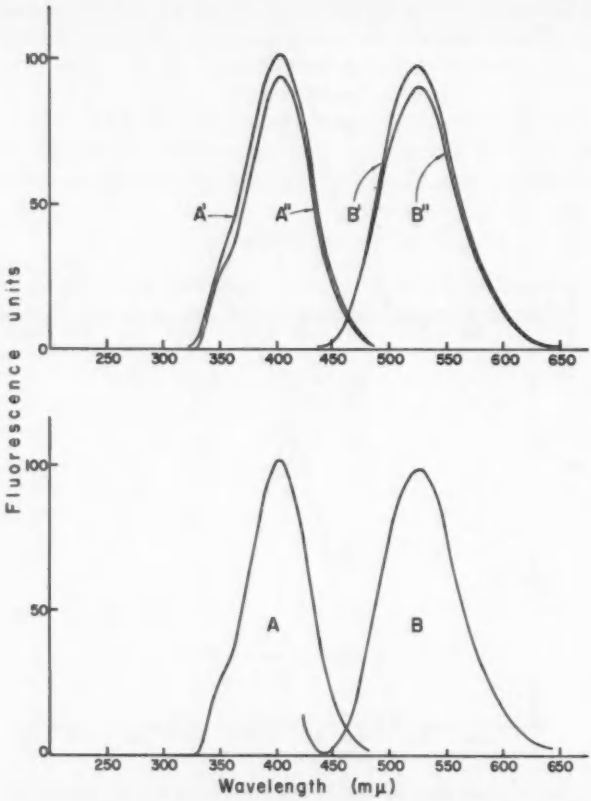

FIG. 3. Excitation (A) and fluorescence (B) spectra of noradrenolutine prepared from noradrenaline (A and B), from trout blood (A′ and B′), and from anterior kidney (A″ and B″). For preparation of excitation spectra, readings of fluorescence were made at 526 mμ; for fluorescence spectra, excitation was at 403 mμ.

1960; Nandi, 1962), the concentrations of the catecholamines were about equal and much higher than those in blood plasma. The concentrations were very variable in rested fish, however, and there was no convincing evidence for any change in concentration in response to disturbance, The concentrations of AD and NAD (0.43 and 0.18 μg/g respectively) found in a kidney sample taken from the region beneath the dorsal fin were much lower than those found in the anterior kidney.

Three experiments similar to those recorded in Table II, but omitting the anterior kidney, and extended to include the liver, heart, and skeletal muscle and appropriate glucose and glycogen measurements, were carried out. Some representative results are presented in Fig. 4–9.

TABLE I. Catecholamine concentrations in blood plasma and anterior kidney of rainbow trout at rest and after various periods of disturbance.

Condition of fish	No. of fish	Catecholamine concentrations in Blood plasma AD	Blood plasma NAD	Anterior kidney AD	Anterior kidney NAD
		μg/ml	μg/ml	μg/g	μg/g
Rested	2	0.006	0.003	3.75	1.93
	2	0.002	0.002	4.86	6.71
	3	0.005	0.002	1.32	2.03
	3	0.005	0.003	4.96	5.71
Disturbed 2 min	2	0.029	0.003	2.00	3.18
10 min	2	0.165	0.042	4.31	4.96
15 min	2	0.208	0.053	5.25	7.23
20 min	2	0.156	0.029	4.11	4.80
30 min	3	0.124	0.029	6.73	6.85
60 min	3	0.120	0.010	5.39	6.34
120 min	3	0.120	0.009	5.05	5.47
Average values – rested fish		0.005	0.003	3.72	4.10
– disturbed fish		0.132	0.025	4.69	5.55

In blood (Fig. 4) the changes in catecholamine concentrations were similar to those described above, but the resting concentrations were much greater and the maximum concentrations somewhat greater in this particular experiment. The reason for these differences is not known, but presumably the "rested" fish were subjected to some unrecognized stress. The maximum concentrations of AD observed in response to disturbance were remarkably high, and spectral evidence of the reality of the values is presented in Fig. 5. In the particular experiment described in Fig. 4, blood glucose concentrations increased slightly during disturbance, then decreased slowly during recovery. In one experiment, the concentration of blood glucose, which was 75 mg % at rest, continued to increase well into the recovery period, reaching a maximum value of 205 mg % after 6 hr and remaining near that value for the first 24 hr of recovery, then decreasing to 115 mg % in the next 24 hr. The difference between these two experiments was associated with a large difference in resting concentrations of liver glycogen and will be returned to below.

The concentration of AD in liver increased during disturbance, then decreased during recovery reaching its approximate resting value within 48 hr (Fig. 6). Liver concentrations of NAD were variable. In the experiment of Fig. 6, liver glycogen concentration did not appear to change consistently. However, in one experiment, the resting value was much higher (7.6%) and there was an apparent fairly steady rate of reduction in concentration during

TABLE II. Catecholamine concentrations in blood plasma and anterior kidney of rainbow trout at rest and after disturbance and recovery.

Condition of fish	No. of fish	Catecholamine concentrations in: Blood plasma AD	Blood plasma NAD	Anterior kidney AD	Anterior kidney NAD
		μg/ml	μg/ml	μg/ml	μg/ml
Rested	2	0.003	0.004	5.09	4.36
	2	0.003	0.003	7.53	5.08
	2	0.004	0.006	5.15	5.51
Disturbed 10 min	2	0.115	0.039	5.47	4.51
10 min	3	0.102	0.024	6.13	3.91
10 min	3	0.120	0.029	4.75	4.26
10 min, 1-hr recovery	2	0.044	0.008	4.43	6.84
10 min, 1-hr recovery	2	0.049	0.025	3.41	5.36
10 min, 1-hr recovery	2	0.032	0.007	4.83	4.32
10 min, 3-hr recovery	2	0.019	0.006	1.21	0.93
10 min, 3-hr recovery	3	0.018	0.004	2.86	3.96
10 min, 3-hr recovery	3	0.019	0.009	4.81	3.02
10 min, 15-hr recovery	2	0.007	0.003	4.89	5.51
10 min, 24-hr recovery	2	0.003	0.003	3.98	2.67
10 min, 24-hr recovery	3	0.004	0.003	6.63	4.41
Average values – rested		0.003	0.004	5.92	4.98
– disturbed 10 min		0.112	0.031	5.45	4.23
– disturbed 10 min, 1-hr recovery		0.042	0.013	4.22	5.50
– disturbed 10 min, 3-hr recovery		0.019	0.006	2.96	2.64
– disturbed 10 min, 15-hr recovery		0.007	0.003	4.89	5.51
– disturbed 10 min, 24-hr recovery		0.003	0.003	5.30	3.54

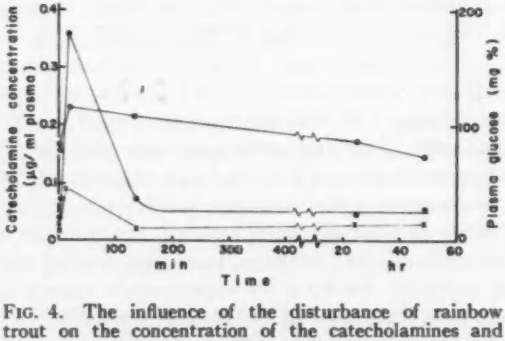

FIG. 4. The influence of the disturbance of rainbow trout on the concentration of the catecholamines and glucose in their blood. Fish undisturbed at time 0, continuously disturbed from time 0 to 15 min, undisturbed thereafter. Adrenaline, ●——●; noradrenaline, ×——×; glucose, ○——○.

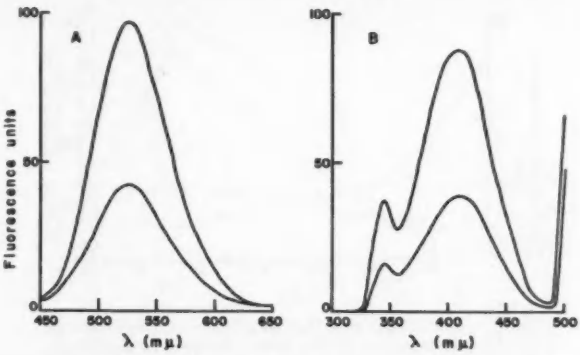

FIG. 5. Fluorescence (A) and excitation (B) spectra of adrenolutine prepared from 1.2 ml of rainbow trout plasma (upper curves) and 0.2 μg adrenaline (lower curves). Sensitivity settings of the fluorimeter the same for all determinations. For preparation of the fluorescence spectra, excitation was at 410 mμ; for excitation spectra, readings of fluorescence were made at 520 mμ. The plasma was the same as that used in the determinations recorded at 15 min in Fig. 4.

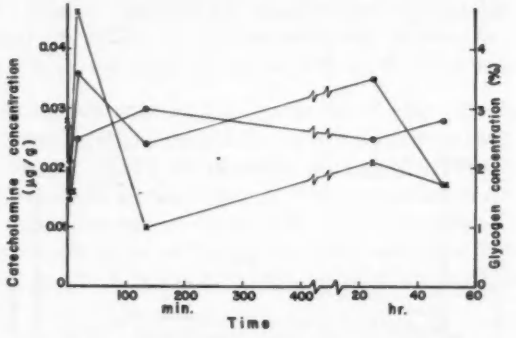

FIG. 6. The influence of the disturbance of rainbow trout on the concentration of the catecholamines and glycogen in their livers. Treatment of fish as in Fig. 4. Adrenaline, ●——●; noradrenalene, ×——×; glycogen, ○——○.

disturbance and the early portion of the recovery period, a minimum value of 1.5% being found after 2 hr of recovery. After 48 hr of recovery, the value had risen to 7.2%. In this latter experiment, a much greater and longer lasting increase in blood glucose was observed in response to disturbance than occurred in the experiment of Fig. 4 and 6 (see above).

In the heart, the concentration of AD increased in response to disturbance, then decreased during recovery (Fig. 7). The decrease in NAD shown did not occur in one experiment. The concentration of glycogen in the heart decreased

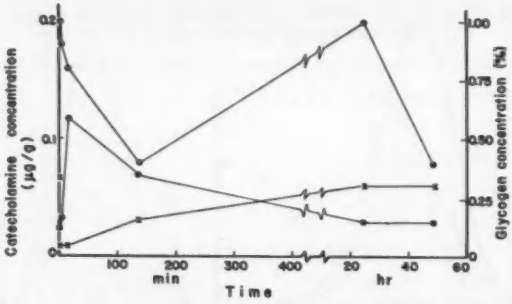

FIG. 7. The influence of the disturbance of rainbow trout on the concentration of the catecholamines and glycogen in their hearts. Treatment of fish as in Fig. 4. Adrenaline, ●——●; noradrenaline, ×——×; glycogen, ○——○.

during disturbance and the early part of the recovery period, then tended to increase thereafter. However, in one experiment in which the resting value was low (0.1%), there was little change, the minimum value observed being 0.07% after 2 hr of recovery, and the final value after 48 hr being 0.09%.

The catecholamine concentrations of skeletal muscle did not change appreciably in response to disturbance (Fig. 8). Although there appeared to be a small increase in NAD in this particular experiment, it was not observed

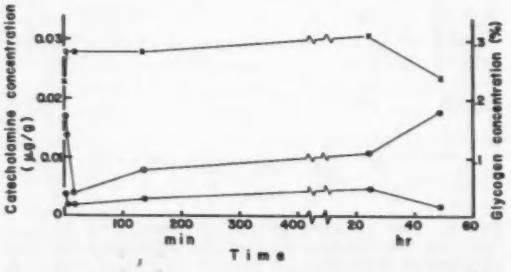

FIG. 8. The influence of the disturbance of rainbow trout on the concentration of the catecholamines and glycogen in their skeletal muscle. Treatment of fish as in Fig. 4. Adrenaline, ●——●; noradrenaline, ×——×; glycogen, ○——○.

in the other two, and the quantity of AD present was so small that its measurement would be unreliable. The concentration of skeletal muscle glycogen fell rapidly during disturbance and increased slowly during recovery. In one experiment, the concentration of cyclic AMP and the degree of activation of glycogen phosphorylase in the skeletal muscle were also examined (Fig. 9). In our hands the assay of cyclic AMP was not completely satisfactory (e.g.,

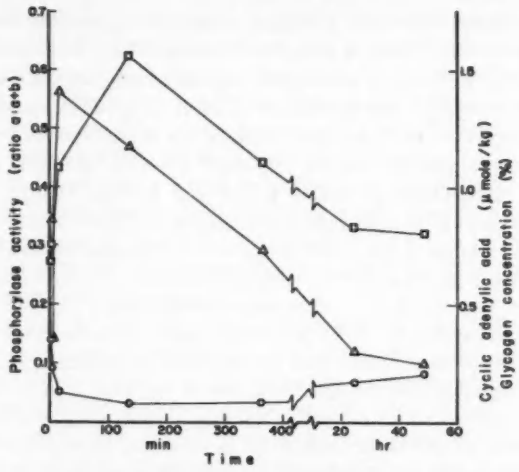

FIG. 9. The influence of the disturbance of rainbow trout on the concentration of cyclic AMP and glycogen and on the activation of phosphorylase in their skeletal muscle. Treatment of fish as in Fig. 4. Cyclic AMP, △——△; phosphorylase activity, □——□: glycogen, ○——○.

the response to known additions of cyclic AMP was not linear) and consequently the values recorded should be regarded with caution. However, they indicate an increase in concentration of the compound in response to disturbance of the fish and a decrease during recovery. The proportion of glycogen phosphorylase present in the muscle in the *a* form increased during disturbance and apparently continued to do so during the early stages of recovery but thereafter decreased, approximating the initial value in about 24 hr.

DISCUSSION

The concentrations of catecholamines found in anterior kidney and kidney of rested trout differed somewhat from those reported earlier for another teleost fish, the Atlantic cod (Östlund, 1954; Euler and Fänge, 1961). In trout anterior kidney, AD and NAD were present in more or less equal concentrations, whereas in cod, AD accounted for practically all of the catecholamines found, and was present in a concentration several-fold greater than that of the combined catecholamines in trout. The kidney of trout contained about the same concentration of AD as that of cod, but a somewhat higher concentration of NAD. The difference in the ratios of the catecholamines in the anterior kidney of the two species is not in accordance with expectations based on a possible relationship between that ratio and the degree of intermingling of interrenal and chromaffin tissues (Coupland, 1953; Wright and Chester Jones, 1955),

since in both Salmonidae and Gadidae chromaffin cells do not occur in the region where interrenal tissue is situated (Nandi, 1962). The ratios in trout were similar, however, to those in interrenal organs of dogfish and skate (Östlund, 1954) and in chromaffin cells of dogfish (Euler, 1953; Euler and Fänge, 1961). The concentrations of the catecholamines in the heart and liver of rested trout were within the ranges previously reported for cod, salmon, pike, and pike perch (Euler, 1953; Östlund, 1954; Euler and Fänge, 1961), but in skeletal muscle they tended to be a little lower than those found in a cyclostome (hagfish) (Euler and Fänge, 1961) and in certain mammals (Sedvall, 1964). However, the relationship between the concentrations of NAD and AD in trout skeletal muscle was similar to that previously found in the other animals, AD accounting for only about 10% of the total catecholamines. Insofar as the concentrations of the catecholamines in the blood of rested trout are concerned, we know of no comparable data for fish, but it appears that although they are greater than those found in man (Cohen and Goldenberg, 1957; Price, 1959), the concentration of NAD is similar to that found in the monkey, although that of AD appears to be somewhat greater (Mason et al., 1961). Since the influence on the blood concentrations in trout of the brief struggle during catching and killing of the fish is unknown, the significance of these latter differences is uncertain.

Disturbance of the fish resulted in an increased concentration of AD and NAD in blood and of AD in heart and liver, but there is no clear indication of the source of these increases. If the major source was in the anterior kidney, then the quantities of catecholamines released into the blood were either too small to be detected by measurement of their concentration in the anterior kidney because of the large variability in these concentrations, or the rate of their production was such that it equalled the rate of secretion. The reduction in the concentration of NAD in heart in response to disturbance suggests that, as in mammals (Vendsalu, 1960; Carlsson et al., 1964; Axelrod, 1965), the sympathetic system may be the source of part of the increase in this compound observed in the blood of trout.

The maximum concentrations of catecholamines observed in the plasma of trout after disturbance (AD, 0.2–0.36 μg/ml; NAD, 0.05–0.09 μg/ml) were higher than those that have been found in the blood of man after death preceded by a state of stress (AD, 0.02–0.09 μg/ml; NAD, 0.008–0.025 μg/ml) or in one instance of fatal poisoning caused by erroneous intravenous injection of AD (AD, 0.051 μg/ml) (Lund, 1964). It would be of interest to know the tolerance of trout to the catecholamines, if there are differences in this respect between different species of fish, and if catecholamines might contribute to the death of certain fish after severe exertion (Black, 1958; Parker et al., 1959; Drummond and Black, 1960).

In general, the changes in plasma glucose and in liver and muscle glycogen concentrations observed in the present investigation during the disturbance and subsequent recovery of rainbow trout resemble those that have been described earlier in fish by others (see review by Black et al., 1961). The apparent association between the magnitude and duration of hyperglycemia and

the initial reserves of liver glycogen, however, does not appear to have been recorded previously, but would be anticipated in view of the relation between degree of hyperglycemia and the nutritional state of fish (Black et al., 1961; Motelica, 1961). Changes in liver glycogen concentrations in response to disturbance or severe exertion have not always been detected. This was also the case in the present work, perhaps because of variability in liver glycogen concentrations in those fish in which initial concentrations were relatively low, for in experiments with fish in which initial and final concentrations were high, a decrease in response to disturbance appeared to occur. Changes in heart glycogen concentrations in fish in response to severe exertion do not appear to have been investigated previously.

The demonstration that the concentrations of AD and NAD in blood and of AD in heart and liver of trout increase in response to disturbance, together with the earlier findings of others regarding the influence of injected catecholamines on carbohydrate concentrations in fish (see review by Plisetskaia, 1964), and the sensitivity of fish hearts to perfused AD (Östlund, 1954), provides direct evidence to support the view that the changes observed in carbohydrate concentrations in blood, heart, and liver of fish in response to disturbance are mediated, in part at least, by the circulating catecholamines. But, since no detectable change occurred in the catecholamine concentrations of the skeletal muscle, there is no similar direct evidence that the circulating hormones play a part in glycogenolysis in this muscle. In addition, while it has been shown that in cyclostomes and skates the intraperitoneal injection of AD results in a reduction in their skeletal muscle stores of glycogen (Plisetskaia et al., 1964), the effect of such injection seems to be much slower to develop than does that of disturbance in trout. However, the rates of change of concentrations of cyclic AMP and of conversion of phosphorylase *b* to *a* in trout muscle in response to disturbance seem to be more in accord with those observed in rat and frog muscle in response to intracardiac injection with, or to in vitro incubation in, AD solutions, than with those observed in response to electrical stimulation (Danforth et al., 1962; Posner et al., 1964).

The observed increase in the muscle concentration of cyclic AMP in trout serves to confirm the finding of Sutherland et al. (1962) that adenyl cyclase activity occurs in fish.

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Extraction and Some Properties of Adenosine 5′-Monophosphate Aminohydrolase from Prerigor and Postrigor Muscle of Cod[1]

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ABSTRACT

Adenosine 5′-monophosphate aminohydrolase (EC 3.5.4.6) activity of prerigor cod muscle could be extracted with water or 0.02 M succinate buffer in about 90% yield, but 0.02 M KCl gave a low yield. With postrigor muscle, the enzyme tended to be associated with the fibrillar protein fractions. The activity of the unpurified enzyme, measured by a *p*H-stat, showed a maximum at *p*H 7.0 in 0.02 M succinate, and about *p*H 6.6 in 0.1 M KCl. At *p*H 7.0, activities were maximal and approximately equal in 0.1 M KCl, 0.1 M NaCl, and 0.04 M potassium succinate. Phosphate buffer inhibited the reaction under certain conditions. The enzyme was sensitive to glass and other surfaces, and this seemed to be responsible for loss of activity during dialysis. The activation energy was 10 kcal per mole and the Michaelis–Menten constant was 1.4×10^{-3} moles per litre.

INTRODUCTION

Jones and Murray (1957) showed that inosine monophosphate (IMP)[2] occurred in cod muscle, and that its concentration first increased and then decreased during postmortem storage of the fish in ice. This substance, important for its flavor-enhancing properties (Kuninaka et al., 1964) was presumably produced from AMP by the action of the enzyme 5′-AMP-aminohydrolase (EC 3.5.4.6., "AMP-deaminase," "adenylic deaminase"). Because of this importance, and also because of some discrepancies found in studies of the ATPase activity of extracts containing the fibrillar proteins of cod muscle, we have examined various extracts for AMP-aminohydrolase and other nucleotide-degrading enzymes that might be present. We report here some properties of AMP-aminohydrolase in extracts of cod muscle, both prerigor and postrigor.

Schmidt (1928) first described 5′-AMP-aminohydrolase from rabbit muscle, and it has since been reported to occur in skeletal muscle of a number of other species, as well as in various other tissues (see review by Lee, 1960). Among marine animals, activity of this enzyme in aqueous and 0.5 M KCl extracts of the muscle of carp and mackerel was measured by Hidaka and Saito (1960), it was prepared in purified form from carp by Nara (1960), and

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[2]Abbreviations used: AMP, ADP, ATP, adenosine 5′-mono-, di-, and triphosphate, respectively; IMP, inosine 5′-monophosphate; P_i, inorganic phosphate.

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Tarr and Comer (1964) found it in aqueous extracts of lingcod muscle, frozen or in rigor. On the other hand, Kitagawa and Tonomura (1957) could not detect any AMP-aminohydrolase activity in extracts of the muscle of *Pecten yessoensis*, Saito et al. (1958) reported that AMP was not deaminated by squid muscle, and Arai (1966) found that several other marine invertebrates also lacked the enzyme. Kutscher and Sarreother (1948) determined the relative activities of the enzyme in muscles of various species, but apart from the work of Hidaka and Saito (1960), no comparable data on yields in various extraction procedures appear to be available.

MATERIALS AND METHODS

Fish

Live cod (*Gadus morhua*, 50–60 cm long), from the local inshore fishery were held in seawater aquaria (6–8 C) at the laboratory. They were killed by a blow on the head, bled by cutting off the tail, and filleted at once. The skinned fillets were stored in polyethylene bags buried in ice. Prerigor extractions were begun within an hour of killing; postrigor extracts were made from fillets that had been stored at 0 C for 1 week.

Extraction

Extractions were carried out in a cold room (2–3 C) with pre-cooled equipment and partially frozen solutions. Samples (approximately 22.5 g) were cut longitudinally from the thick midportion of the fillets. Low ionic strength (low-μ) extracts were made by homogenizing the sample for 90 sec with 440 ml of either (*a*) water, (*b*) 0.02 M K-succinate buffer, *p*H 6.6, or (*c*), unbuffered 0.02 M KCl in a Waring blendor operated at 90 v and fitted with a baffle plate (Dyer et al., 1950). The supernatant solution was recovered after centrifuging the extract for 15 min at 8000 $\times$ *g*, and was usually recentrifuged for 1 hr at 105,000 $\times$ *g* in a Spinco model L ultracentrifuge. To make myofibrillar extracts, the residue from the first centrifuging of a low-μ succinate extract was reblended with 410 ml of the same extracting solution, and the centrifuged residue from this washing reblended with 410 ml of 0.60 M KCl. The final mixture was centrifuged for 15 min at 8000 $\times$ *g*. Total soluble protein (TSP) extracts were made by homogenizing the sample in the blendor with 440 ml of 0.60 M KCl for 90 sec and then centrifuging at 8000 $\times$ *g* for 15 min. All extracts were stored in glass at 0 C.

No attempts were made to achieve exhaustive extractions by repetitive procedures. Representative yields of protein nitrogen actually recovered (expressed as per cent of the wet weight of the sample) were as follows: prerigor ultracentrifuged low-μ extracts in 0.02 M K-succinate or 0.02 M KCl, 0.41%; in water, 0.58%; prerigor myofibrillar extracts, 0.82%; and prerigor TSP extracts, 1.48%. The corresponding postrigor extracts yielded, respectively, 0.33%, 0.41%, 1.47%, and 1.96%. Dyer et al. (1957) reported that the average total protein nitrogen content of cod muscle is 2.48% (wet weight).

Reaction Procedures

Several procedures for measuring the reaction were used. In one of these, a solution containing the desired concentrations of AMP (Schwarz Bioresearch Inc.) buffer, and other substances as required, was added to appropriately diluted extract. After thorough mixing, the reaction proceeded without stirring at room temperature (20–25 C), which remained constant within 0.2 C throughout a given series of determinations. At intervals, aliquots were withdrawn, mixed with nine volumes of cold 3.3% perchloric acid, and allowed to stand at 2 C for not less than 2 hr. The precipitates were removed by centrifuging, and portions of the supernatant solutions treated with equal volumes of cold KOH of a concentration to give approximate neutrality. The KOH solution also had 0.5 M K-phosphate buffer, *p*H 7.0, to assure that the final mixtures were at this *p*H. After removal of the potassium perchlorate crystals by centrifuging, the ultraviolet absorptions of the solutions were measured against appropriate blanks with a Beckman DK-2 spectrophotometer. The concentrations of inosine-type compounds (assumed to be IMP) were calculated from absorbancies at 260 mμ and 240 mμ, using the molar absorbancy indices determined in phosphate buffer, *p*H 7.0, for AMP (a_M at 260 mμ = 15.8 $\times$ 10^3, a_M at 240 mμ = 6.00 $\times$ 10^3) and for IMP (a_M at 260 mμ = 7.85 $\times$ 10^3, a_M at 240 mμ = 10.5 $\times$ 10^3). Both of these materials were supplied by Schwarz Bioresearch Inc. Results were expressed as per cent of total nucleotides, determined from the absorbancy at the isobestic point, 251 mμ (a_M = 12.8 $\times$ 10^3).

In determination of ammonia formed in the reaction as a function of time, a series of identical reaction mixtures was used. The reactions were stopped by addition of one-ninth volume of cold 30% perchloric acid, and subsequently treated as above. Ammonia was determined either by direct Nesslerization of the supernatant solutions or after separation of the ammonia by the micro-Conway procedure of Öbrink (1955) (Conway, 1951).

The reaction was also followed by a *p*H-stat (Radiometer Titrimeter, type TTTla). Extract was diluted as necessary just before use, then 5.00 ml was added to 45.0 ml of a solution containing the AMP and any other desired substances. The mixture was stirred magnetically in a beaker immersed in a water bath by which the temperature could be maintained within +0.2 C. Automatic addition of 0.01 N HCl was limited to 5 ml to avoid excessive dilution of the reactants. To check the validity of this procedure, samples were withdrawn from the mixture and analysed by ultraviolet spectrophotography as above. After corrections for the changes in volume caused by the withdrawal of samples, the extents of reaction indicated by the two procedures agreed within 3%. The *p*H-stat worked very well in the absence of any buffer. It was found to be satisfactory also in the presence of 0.02 M succinate buffer at *p*H values greater than 6.5, but could not be used when phosphate buffer was present.

The per cent reaction was usually a linear function of time up to about 30% consumption of AMP, and it was possible to estimate the activity of the sample from the initial slopes of the curves with an error of 10% or less.

Protein concentrations are given as milligrams of N per milliliter, and were measured by the biuret method of Snow (1950). Phosphate was determined by the method of Rockstein and Herron (1951).

RESULTS

Reactions with Various Substrates

The difficulties involved in measuring the ATPase activity of a postrigor myofibrillar extract are illustrated by Fig. 1. The amount of inorganic phosphate (P_i) produced in the presence of 1.5 mM ATP exceeded that expected

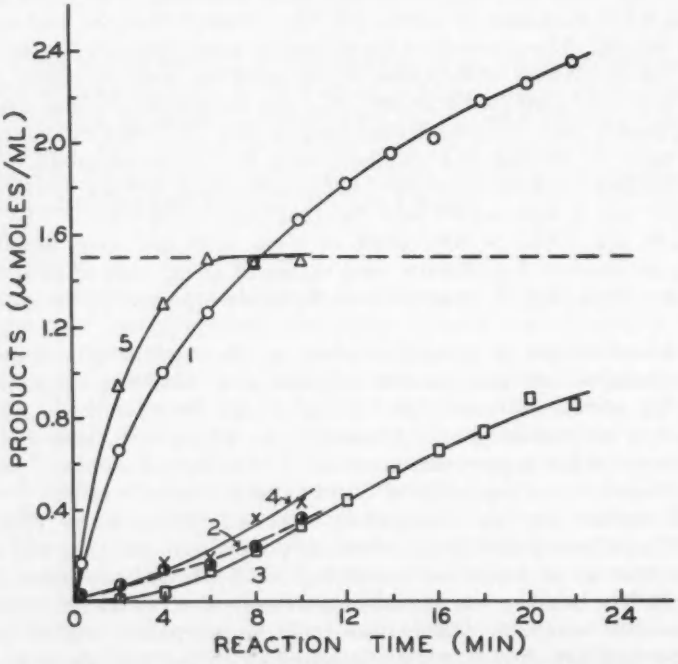

FIG. 1. Rate of product formation from adenine nucleotides in the presence of myosin B extract of postrigor cod muscle. Nucleotides, 0.015 M; $MgCl_2$, 0.015 M; imidazole buffer, pH 6.8, 0.05 M; total ionic concentration ~0.12; 25 C. Curves 1 and 2: inorganic phosphate from ATP and ADP respectively; curves 3, 4, and 5: inosine-type compounds from ATP, ADP, and AMP, respectively. Adenosine gave no products.

for the ATPase reaction after 8 min, and appeared to be approaching twice this value. Moreover, a deamination occurred after an initial delay, and the amount of inosine-type compounds formed agreed with the excess P_i at 22 min. In the presence of 1.5 mM ADP, both P_i and inosine-type compounds

were formed without delay, and at rates comparable with the production of the latter in the presence of ATP. AMP was rapidly deaminated, but adenosine gave no reaction. These results suggest the presence of myokinase and AMP-aminohydrolase as well as ATPase, and that the myokinase reaction is considerably slower than the other two. It also appears that the slowness of the myokinase reaction would permit a reasonably accurate assessment of the ATPase rate to be made, if this were confined to the initial stages.

An ultracentrifuged prerigor extract made with 0.02 M succinate was similarly tested (Fig. 2). AMP was rapidly deaminated, ADP was deaminated

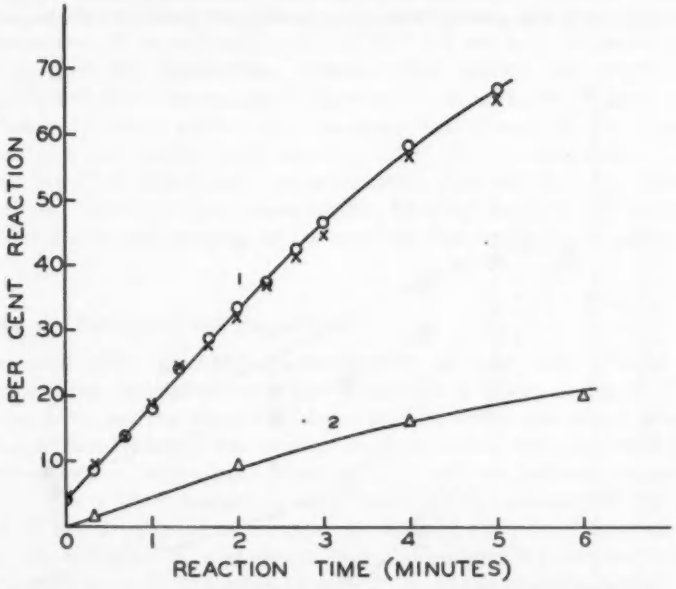

FIG. 2. Deamination of AMP and ADP by ultracentrifuged 0.02 M succinate extract of prerigor cod muscle. Protein concentration, 0.0095 mg N/ml; 0.02 M succinate buffer, *p*H 7.0; 25 C. Curve 1: AMP substrate, 2.7×10^{-3} M; ○, inosine-type compounds from ultraviolet absorption; ×, ammonia from Conway assays. Curve 2: ADP substrate, 2.4×10^{-3} M; inosine-type compounds.

much more slowly, and ATP was not deaminated at all. There was no dephosphorylation with any of the nucleotides up to 10 min. The extract therefore appeared to be free of myosin (ATPase), and if AMP- or IMP-dephosphorylases were present, their activities were negligible. The deamination of ADP probably resulted from the combined action of myokinase and AMP-aminohydrolase, although the presence of ADP-aminohydrolase cannot be ruled out. Figure 2 also illustrates, for the AMP reaction, the equimolar formation of inosine-type compounds and NH_3, as would be expected for the deamination

of AMP. The absence of any deamination when ATP was the substrate supports the present belief that an enzyme capable of converting ATP directly to ITP is lacking in most fish muscle (Jones and Murray, 1960).

Stability

The AMP-aminohydrolase activity was found to be sensitive to several conditions, which dictated precautions observed in measuring its properties. The activity of a crude prerigor extract in 0.02 M succinate decayed to about 50% of its initial value during storage for 3 days at 0 C; thereafter the loss of activity was more gradual (Fig. 3). This initial rapid decay did not occur, however, if the crude extract was ultracentrifuged just after preparation, and

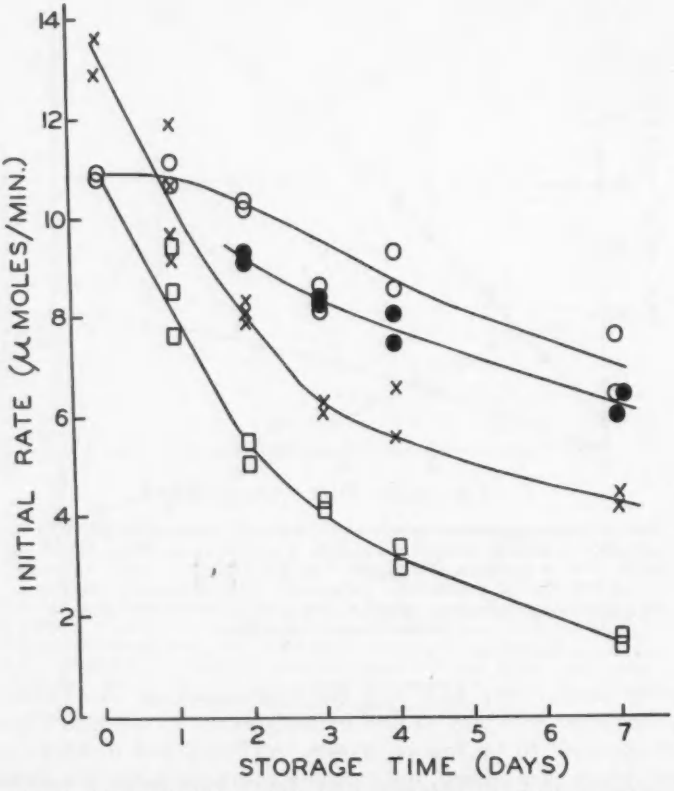

Fig. 3. Decay of AMP-aminohydrolase activity in a 0.02 M succinate extract of prerigor cod muscle. Extract stored at 0 C. Reaction followed by pH-stat at 25 C and pH 7.0 in the presence of 0.1 M KCl; extract diluted 1:75. ×, crude extract; □, crude extract ultracentrifuged just before assay; ○, extract ultracentrifuged, then stored at 0 C; ●, same as ○, but re-ultracentrifuged just before assay.

this procedure was consequently used in most experiments with prerigor low-μ extracts. In some experiments, but not in all, such ultracentrifuged extracts were found to suffer loss of activity, even at 0 C, if allowed to stand after being diluted with water, preparatory to adding them to the reaction mixture. Therefore, necessary dilutions were made only just before the reaction was started. Dialysis of a similar extract in cellulose tubing (Visking Co.) against 0.02 M succinate buffer for 18 hr resulted in a loss of about 50% of the activity. The presence of 10^{-4} M ATP, AMP, or IMP in the dialyzing solution did not prevent this loss. When small pieces of the tubing were added to the undialyzed extract, a similar loss occurred, so that the enzyme seemed to be adsorbed or destroyed at the cellulose surface. In one experiment, however, dialysis of a postrigor myosin B extract against 0.6 M KCl did not lead to loss of activity, suggesting that the myofibrillar proteins may protect the enzyme. Some AMP-aminohydrolase preparations appeared to be sensitive to glass surfaces. When one such low-μ extract, for example, was diluted in the presence of glass beads (4 mm diam, total area 160 cm^2) the activity within 1 min of dilution was 60% of the control, and continued to decrease upon standing. Other preparations, however, were more stable. Flowing nitrogen or oxygen over the surface during the progress of the reaction had no significant effect on the activity.

Activity vs. Protein Concentration

The dependence of AMP-aminohydrolase activity upon protein concentration for three types of prerigor low-μ extracts is shown in Fig. 4. Although the shape of the curves was somewhat variable, in no case was it possible to extrapolate them through the origin. This may have been caused by loss of the enzyme at the fresh glass surfaces of the reaction beakers, or possibly to the presence of a small amount of some inactivating substance in the reaction mixture. If one attempted to measure the specific activity of a sample at low protein concentration, it is apparent that a considerable error could be introduced. Consequently, the average slopes of the initial reaction rate vs. protein concentration curves were used when estimating the total enzyme activity in an extract. In other cases, such as dependence of activity on *p*H, ionic strength, etc., relative values obtained at one sample dilution were deemed sufficient.

Influence of Extraction Conditions on Yields of Enzyme

The variation in yield of enzyme with type of extracting solution and state of the muscle is shown in Table I. Results from other experiments, made for other purposes, support these data but are not directly comparable because of biological variations and other differences. Of the low-μ extractants, water generally gave the greatest total yield and yield per gram of muscle, but the yield per unit of protein in the extract was usually greater in the 0.02 M succinate extracts. The prerigor low-μ extracts were about four times as rich in enzyme as the corresponding post-rigor ones; in the transition to the postrigor state,

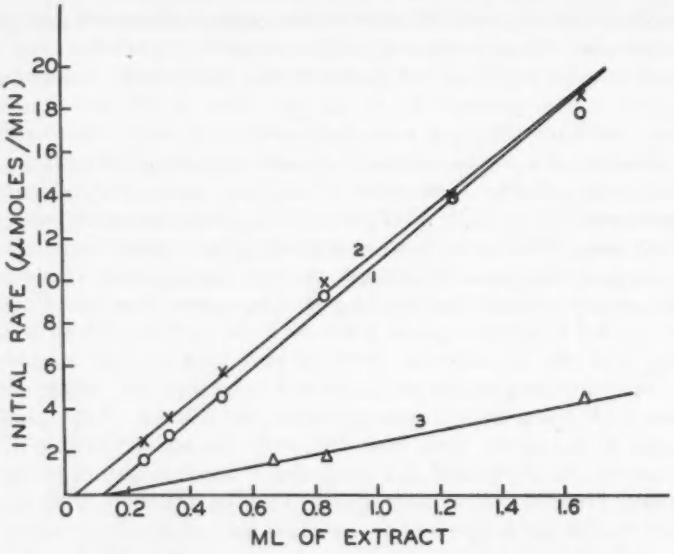

FIG. 4. Effect of concentration of ultracentrifuged low-μ extracts of prerigor cod muscle on deamination of AMP. Reaction followed by pH-stat; AMP, 1.2×10^{-3} M; KCl, 0.10 M; pH 7.00 (no added buffer); 25 C. Curve 1: 0.02 M succinate extract, 0.171 mg N/ml; curve 2: water extract, 0.297 mg N/ml; curve 3: KCl extract, 0.180 mg N/ml. Total reaction volume, 50 ml.

TABLE I. Effect of extracting solution and postmortem state of muscle on yield of AMP-aminohydrolase.

Extract	Units[a] per gram of muscle			
	Prerigor			Postrigor
Low-μ, ultracentrifuged				
Expt. No.	42	43	51	–
Water	210	319	310	–
0.02 M succinate	188	439	225	–
0.02 M KCl	43	102	68	–
0.02 M succinate				
Crude		520		133
Ultracentrifuged		385		95
Myosin B, crude		33		121[b]
TSP, crude		379		325

Reaction followed by pH-stat. The data in the lower part of the table were obtained from the muscle of one fish, before and after passing through rigor at 0 C.

[a]One unit of activity is defined as the amount of enzyme which will deaminate 1 μmole of AMP per minute in the presence of 1.2 mM AMP and 0.1 M KCl, at pH 7.0 and 25 C.

[b]Known to be a poor extract (low-speed blending).

the enzyme appeared to become associated with the myosin fraction. The remarkably low yield in prerigor 0.02 M KCl extracts was paralleled in other experiments.

Dependence of Activity on Ionic Concentration

The activities of prerigor extracts in water and in 0.02 M succinate were measured with the *p*H-stat at 25 C and *p*H 7.00 in the presence of various concentrations of KCl, NaCl, and K-succinate (Fig. 5A and B). The results

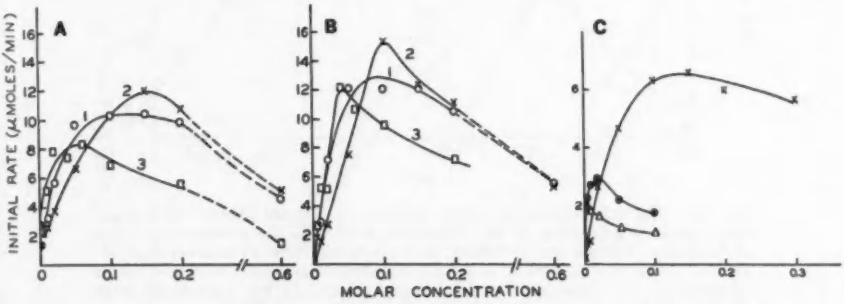

FIG. 5. Effect of salts on AMP-aminohydrolase activities of low-μ extracts of prerigor cod muscle. Reaction followed by *p*H-stat at 25 C and *p*H 7.0. A and B: water and 0.02 M succinate extracts, respectively, in the presence of: ○, KCl; ×, NaCl; □, K-succinate. C: water extract in the presence of: ×, NaCl; △, $MgCl_2$; ●, $CaCl_2$.

with similar postrigor extracts were analogous although these extracts were much less active. In all cases, the activities were greatly enhanced by the addition of these salts to the extracts, and reached maxima between 0.1 and 0.2 ionic concentration. On the other hand, the effects of $MgCl_2$ and $CaCl_2$ were very much less and the maxima occurred at a lower concentration. This is shown in Fig. 5C for a water extract of another prerigor muscle sample; the effect of NaCl on this extract is included for comparison.

Variation of Activity with *p*H

The dependence of activity of a prerigor water extract on *p*H, in the presence of 0.10 M KCl and no added buffer, is shown in Fig. 6. The curve for a similar extract, measured in 0.02 M succinate buffer, is also given. In this case the per cent reaction was estimated both by the *p*H-stat, and by ultraviolet absorption analysis of samples withdrawn after 1 min and 4 min of reaction.

Effect of Temperature

The activity of a prerigor 0.02 M succinate extract was measured in duplicate runs at 25, 20, and 2 C, in 0.10 M KCl and at *p*H 7.00. The Arrhenius plot indicated an activation energy of 10.7 kcal per mole.

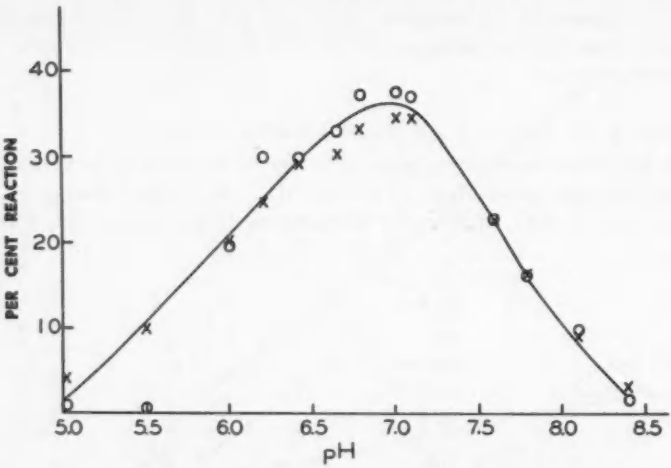

FIG. 6. The AMP-aminohydrolase activity of a water extract of prerigor cod muscle as a function of *p*H. Measured at 25 C in the presence of 0.02 M K-succinate. Protein concentration, 6.68 μg N/ml. Activity expressed as per cent of AMP (1.2×10^{-3} M = 100%) consumed between 1 min and 4 min of reaction. ○, measured by the *p*H-stat procedure; ×, calculated from ultraviolet absorption.

ACTIVITY AS A FUNCTION OF SUBSTRATE CONCENTRATION

With myosin B extracts of postrigor muscle, the initial reaction rate in 0.02 M phosphate buffer tended to approach a saturation value as the substrate concentration was increased. Lineweaver-Burk reciprocal plots indicated a Michaelis-Menten constant (K_m) of 0.7 to 3.0×10^{-3} moles per litre. Postrigor sarcoplasmic extracts, however, behaved erratically, and no useful data were obtained. Prerigor extracts, both sarcoplasmic and TSP, yielded apparently normal saturation curves in the absence of inorganic phosphate, and gave K_m values of 1.6 to 6×10^{-3} moles per litre. The presence of inorganic phosphate caused some inhibition of the reaction, but the effectiveness of the phosphate diminished with increasing substrate concentration, and was also reduced by the addition of KCl (Fig. 7).

DISCUSSION

It seems to have been customary, in preparing muscle AMP-aminohydrolase, to extract the tissue with solutions that also dissolved the myosin. When purification of the enzyme was desired, extra procedures were required to break the apparent strong association between the enzyme and the myosin. Even with rabbit muscle, however, Kalckar (1947) early showed that not only could the enzyme be extracted with water, but that the yields were considerably better than when prepared from extracts containing myosin. Our results, and those of Tarr and Comer (1964) and Hidaka and Saito (1960) suggest that

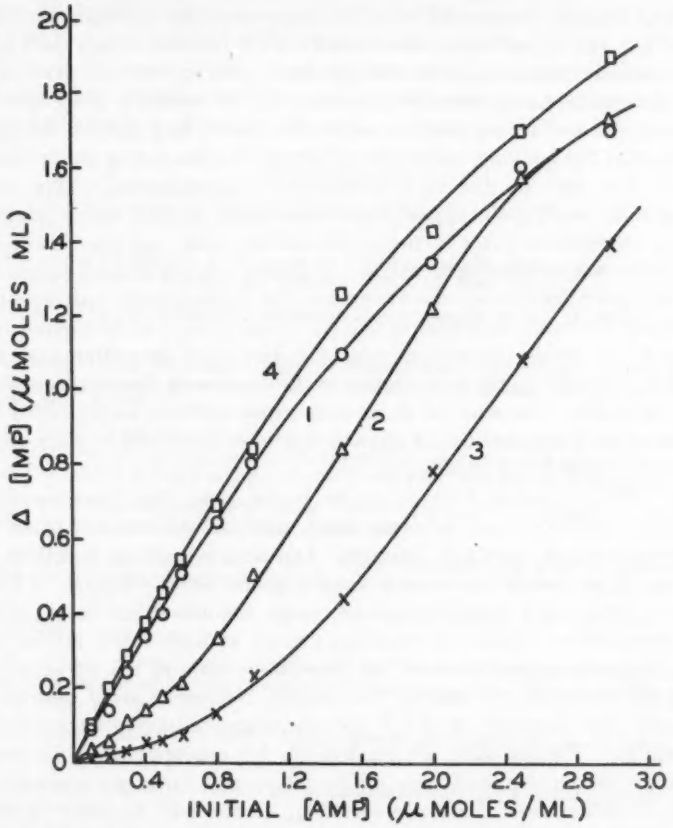

FIG. 7. Effect of substrate concentration and of inorganic phosphate on the activity of AMP-aminohydrolase of prerigor cod. Ultracentrifuged 0.02 M succinate extract; concentration in reaction mixture, 0.0097 mg N/ml. Inosine-type compounds determined from ultraviolet absorption spectra after 3 min of reaction at 25 C and *p*H 7. Curve 1, in 0.02 M succinate; curve 2, in 0.02 M phosphate; curve 3, in 0.12 M phosphate; curve 4, in 0.02 M phosphate and 0.25 M KCl.

fish muscle resembles rabbit muscle in extractability of AMP-aminohydrolase. Hermann and Josepovits (1949) said that it is known that simple extraction of rabbit muscle with water dissolves some myosin, and that this accounts for the presence of AMP-aminohydrolase in such extracts reported by Kalckar (1947). Although we confirmed the finding of Fraser and Dyer (quoted by Matsumoto et al., 1967) that water does indeed extract more protein from prerigor cod muscle than do weak salt solutions, we were unable to demonstrate that the "extra" protein in the water extracts was myosin. In any case, the amount of extra protein was only a small fraction of the amount of myosin in the muscle, and yet the extract contained most of the available aminohydrolase

activity. The poor extractability of the enzyme from prerigor muscle when using 0.02 M KCl is puzzling. Water and 0.02 M succinate may perhaps cause swelling and disruption of some membranous structure or cell particle and so release the enzyme, whereas the presence of KCl exerts a protective action. The change in extraction pattern after the muscle has passed through rigor indicates that the enzyme undergoes a change of state during this process also, and this, too, may be due to a breakdown of membranes within the fibre.

The data in Fig. 3 suggest that there may be two decay mechanisms operating at different rates in the crude extract, and that the slower of these occurs in the ultracentrifuged extract. Possibly the rapid mechanism is associated with particles that are removed when the crude extract is ultracentrifuged. If this is so, however, the effect of the particles appears to be limited to 2 or 3 days since the slower decay becomes the dominant one after that time. A possible explanation might be a binding of the enzyme to the particles until these become saturated. The slow decay in both crude and ultracentrifuged extracts seems to be accompanied by an aggregation of some of the protein which can then be separated by recentrifuging.

Lee (1957) reported that succinate buffer gave the "best results" with rabbit muscle AMP-aminohydrolase assay, whereas a number of other buffers, including phosphate, were not "suitable." Our results indicate that the apparent activation by succinate buffer may simply be due to an increase in ionic concentration, since KCl gave comparable rates. On the other hand, phosphate buffer appeared to inhibit the reaction except at high AMP levels, possibly because the competition between the phosphate ion and the phosphate group of the AMP for the active site of the enzyme. Nikiforuk and Colowick (1956) also found that increase of AMP concentration reduced the inhibition by phosphate ion. Present data are inadequate for exploring this effect further.

The rather erratic sensitivity of the enzyme to surfaces appears to have occurred in other work. Liubimova (1957), Currie and Webster (1962), and Tarr and Comer (1964), for example, reported a loss of activity when the enzyme was dialyzed. From Fig. 3 in the paper by Nara and Saito (1959) one can see that the curve of activity vs. protein concentration did not pass through the origin, although the authors did not comment on this behavior, which was similar to our experience with cod AMP-aminohydrolase. Fujimaki and Deatherage (1964) found that the enzyme activity was lost when sarcoplasmic extract of beef muscle was passed through an ion-exchange cellulose. The causes of these phenomena do not seem to have been extensively investigated, although Liubimova (1957) claimed that the activity lost upon dialysis could be partly restored by large concentrations of ATP or ADP, possibly because of some impurity contained in these reagents. This, however, does not rule out the possibility that the original loss of activity was due to some surface phenomenon.

The Michaelis–Menten constant and the *p*H-dependence of the activity of crude AMP-aminohydrolase from cod agree at least roughly with those reported for the enzyme prepared from mammalian muscles. The absence of a

significant adenosine aminohydrolase activity in extracts of cod muscle is another point of resemblance to mammalian muscle (Brady and O'Donovan, 1965). Tarr and Comer (1964), however, did find adenosine aminohydrolase in ling cod muscle extracts, so that a survey of various fish muscles for these two aminohydrolases might be of interest.

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Swimming Performance of Sockeye Salmon (*Oncorhynchus nerka*) in relation to Fatigue Time and Temperature[1]

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ABSTRACT

Further studies on the swimming performance of fingerling sockeye salmon at fixed velocities have been conducted in relation to fatigue time. The method of probit analysis, commonly used in dealing with bioassay data, was found to be suitable for determining times to 50% fatigue and in providing a measure of variance despite the presence of some erratic behaviour. For sockeye acclimated to 15 C (mean length = 13.6 cm) the velocity at which 50% fatigued was 54.4 cm/sec or 4.0 lengths/sec (L/sec). The 5% and 95% fatigue velocities were 3.1 and 4.8 L/sec, respectively. Larger fish required longer exposure times for determining maximum sustained speeds, extending from approximately 120 min for fingerlings to 500 min for adults.

Using the method of increasing velocity steps the effect of temperatures from 5 to 27.5 C was examined. When acclimated to 15 C fingerling sockeye exhibited only a 4% reduction in swimming speed at 10 and 20 C. Temperatures above the lethal level caused a rapid decline in swimming ability approaching the extinction point at 27.5 C.

Recommendations for standard procedures in the study of swimming speeds are made.

INTRODUCTION

Studies on the swimming performance of fish have been made in relation to various environmental factors such as temperature, oxygen, carbon dioxide, and toxic solutions (Fry and Hart, 1948; Katz et al., 1959; Davis et al., 1963; Herbert and Shurben, 1963; MacLeod and Smith, 1966), size, body form, and movement (Brainbridge, 1958, 1963; Ohlmer, 1964), the physical condition of the fish (Bainbridge, 1962; Thomas et al., 1964), and the duration of the test as well as the recovery time (Bainbridge, 1960; Brett, 1964; Paulik et al., 1957). Different types of apparatus have been used in the above experiments, ranging from troughs and tubes to circular chambers or fish wheels. Observations were made on either cruising speed, maximum sustained speed, or burst speed. These have resulted in a variety of records which are not all readily compared. Differences also exist in the swimming path (straight or circular), possible wall effect, water turbulence, and stimulus to swim.

During studies on the metabolic rate of yearling sockeye salmon in relation to swimming speed a step-type, sequential increase in velocity was imposed, each step lasting for 60 min (Brett, 1964). The test was continued until a state of exhaustion was reached. An attempt was made to relate this measure of performance to that obtained by applying various fixed velocities for test-periods of up to 1000 min (approximately 17 hr). Considerable variability in

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response occurred. Some of the variations obviously resulted from erratic behaviour, whereas others appeared to be the result of excellent physical condition not unlike that of reaching a "second wind." In order to obtain an estimate of mean performance the data were treated in the manner of bioassay tests, using imposed velocity as the *dose* and fatigue time as the *response*. Appropriate transformation (see Fig. 2) provided the time to 50% fatigue (FT_{50}) at any velocity, with a measure of variance determined graphically (Litchfield and Wilcoxon, 1949). Since the number of cases was not large it was desirable to obtain additional data including a greater size range.

Further it had been discovered that sustained performance appeared to be restricted at temperatures above 15 C through the curtailment of any increase in active metabolic rate (Brett, 1964). In this latter study experiments at 20 and 24 C were performed keeping below the known lethal temperature of 24.8 C for young sockeye in freshwater (Brett, 1952).

The capacity to be active above this temperature in the *zone of thermal resistance* (Fry, 1947) has now been examined by conducting swimming tests at temperatures up to 27.5 C on young sockeye acclimated to 15 C. These observations are presented here and compared with the results of previous experiments.

MATERIALS AND METHODS

Freshwater-adapted sockeye salmon (*Oncorhynchus nerka*) were used, either as cultured young fish (total length[2] = 13.6 ± 1.8 cm,[3] weight = 25.3 ± 11.9 g, age = 9–10 months from hatching) or as wild adults (length = 53.2 ± 2.8 cm, weight = 1739 ± 242 g, age = mostly 4 years) taken from the fishway at the outlet of Great Central Lake 4 miles upstream from the sea. These were tested in a tunnel respirometer of 11.4 cm (4.5 inch) diam for the fingerling fish (Brett, 1964, 1965) and in a 28 cm (11 inch) diam water tunnel in the case of the adults (Brett and Shoop, unpublished data). Standard procedures for culturing and handling have been described in the references cited above.

The two techniques for determining maximum sustained speeds require brief recounting.

Fixed velocity. — From a knowledge of the sort of speeds which were likely to result in fatigue (derived from preliminary experiments) a series of velocities was chosen which ranged from those producing rapid fatigue to those at which some or all of the fish might be swimming at the end of the prescribed time. In practice it was impossible to introduce fish directly to a high velocity in an unfamiliar environment. Some sort of introductory period was necessary. The one adopted by experience with this species was a 20–30-min conditioning period at a low velocity (30 cm/sec), followed by a 5-min period at an inter-

[2]The total length used here and throughout the paper is measured from the tip of the snout to a line joining the tips of the tail when the latter are placed in a natural position (not squeezed to maximum extension). For the sockeye fingerlings this total length is about 7% more than fork length; for the adults this difference is about 4½%.

[3]Standard deviation used throughout.

mediate velocity, and then increased to the prescribed level. A low charge (3–5 v a-c) prevented fish from resting on the downstream screen.

Test times of 1000, 600, and 300 min were used, reduced in this order as it became apparent that times in excess of 300 min were not necessary for fingerling fish.

Increased velocity increments. — A slightly modified technique from that adopted for the determination of metabolic rates in relation to activity was used when investigating swimming speeds only. Three to five of the small fish were placed in an extended section of the respirometer tube (fish chamber length increased from 30 to 120 cm) for 0.5 hr at an introductory velocity of 21 cm/sec (0.7 ft/sec) prior to commencing a series of increasing velocity steps, each 9.2 cm/sec (0.3 ft/sec), applied for 45 min. As a given fish fatigued it was carefully removed with forceps from the chamber through a plexiglass well placed in front of the electrified screen; the procedure was continued until the last fish succumbed. Some shortcoming of this technique was apparent on occasion when the act of removing a fatigued fish caused undue excitement among the remainder. Since leaving the fatigued fish on the screen provided an opportunity for one or other of the swimming fish to take advantage of the "bow wave" so formed it was necessary to carry out the removal procedure.

Because the fish almost always fatigued during the last velocity increment in considerably less than the prescribed time-interval a measure of *critical swimming speed* was provided (Brett, 1964). This was obtained empirically by adding that fraction of the velocity increment (0.3 ft/sec) to the last recorded speed (say 2.0 ft/sec) according to the fraction of time sustained (say 15 min). In this hypothetical case the critical swimming speed would be: $2.0 + (15/45 \times 0.3) = 2.1$ ft/sec. This index has been found to provide a close measure of the maximum sustained speed when derived by the fixed velocity technique.

RESULTS

Fixed Velocity Tests

The results of experiments on 104 fingerling sockeye are shown in Fig. 1 and Table I. A typical "dosage–response" curve characterized the relation between imposed velocity and per cent fatigued. For a test time of 300 min, 50% of the fish had fatigued at a swimming speed of 4.0 L/sec. The approximate 5 and 95% fatigue levels occurred at 3.1 and 4.8 L/sec, respectively.

For velocities where 50% or more of the sample succumbed, the logarithm of the individual times to fatigue have been plotted against per cent fatigued on a probit scale (Fig. 2). In general only the points lying beyond ± 1 standard deviation of the mean showed much departure from a straight line. It was apparent that beyond a test time of about 120 min most of the fish that were still swimming had reached a state of sustained performance. Early, abrupt departures from linearity reflect the problem of erratic behaviour in which the collapse of one fish could induce excitement resulting in one or two additional fish being prematurely forced onto the screen, from which they could not escape.

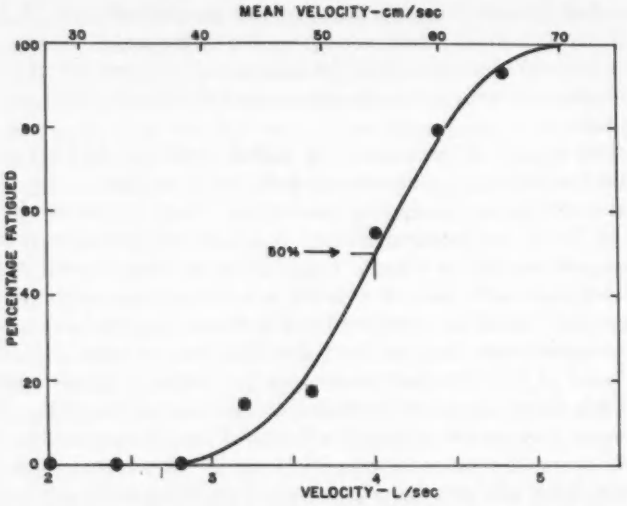

FIG. 1. The percentage of young sockeye salmon fatigued within 300 min at 15 C when forced to swim at the velocity indicated, expressed in lengths/sec (L/sec). Mean length of the sample of 104 fish was 13.6 cm.

TABLE I. The relation of swimming speed to the per cent fatigued and time to 50% fatigued for young sockeye salmon. Total test time was 300 min. Tests were conducted from September 22 to November 6 at a temperature of 15 C. Water velocity has been expressed in terms of fish lengths per second (L/sec). The limits of ±1 SD were determined graphically from Fig. 2.

	Velocity (L/sec ±0.2)							
	2.0	2.4	2.8	3.2	3.6	4.0	4.4	4.8
No. of fish	10	5	3	7	17	29	19	14
Mean length (*cm*)	12.1	14.0	12.4	14.5	14.3	13.4	12.6	13.8
Fatigued (%)	0	0	0	15	18	55	79	93
Time to 50% fatigued (*min*)	–	–	–	>300	>300	66	21	6
Mean +1 SD	–	–	–	–	–	>120	120	30
Mean −1 SD	–	–	–	–	–	9	3	1

If the logarithms of the time to 50% fatigue are plotted against velocity for a series of experiments performed on fish of different size (as well as season and temperature) it is seen that the slopes are almost identical (Fig. 3). There is a difference in performance capacity, mainly according to size, with the smallest showing the greatest *relative* ability to swim and the shortest time

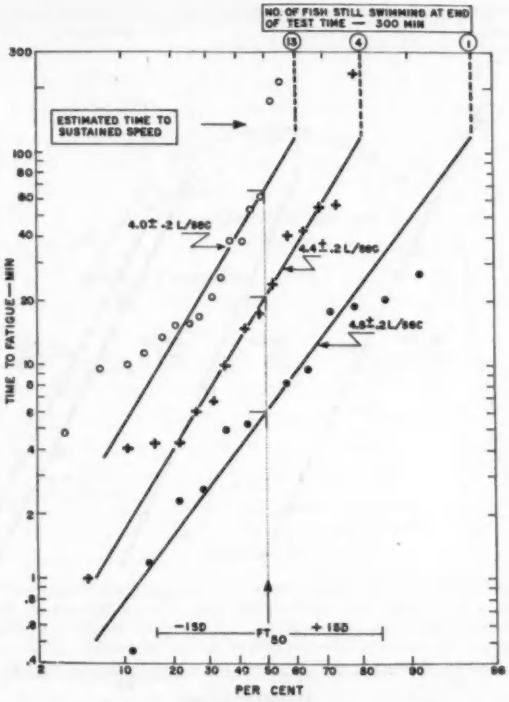

FIG. 2. Relation of fatigue time (log) to order of fatigue (probit) for sockeye fingerlings (13.6 cm, 25.3 g, 15 C) tested at three velocities (L/sec). The estimated minimum time to sustained speed is indicated by the inflection from linearity depicted by the use of broken lines.

to reach a sustained level of performance. The results from 52 adult sockeye indicated that a period of approximately 500 min was necessary before a sustained speed could be established.

The similarity of the slopes despite the different size and history of the fish allows the comparison of tests which differ by some particular time interval. Thus, the difference between the mean velocity maintained for 45 min and that for 60 min is approximately 0.1 L/sec. Since these two time-intervals were used in the step-type velocity tests an estimate of the correction factor is provided.

INCREASING VELOCITY INCREMENTS

The relation of critical swimming speed to test temperature when acclimated to 15 C is seen to be dome-shaped, unlike the "split" curve for fully acclimated sockeye (Fig. 4, Table II). Beyond the temperature tolerance zone a rapid decline in ability occurs showing an incapacity of the fish to perform when injurious lethal temperatures are experienced.

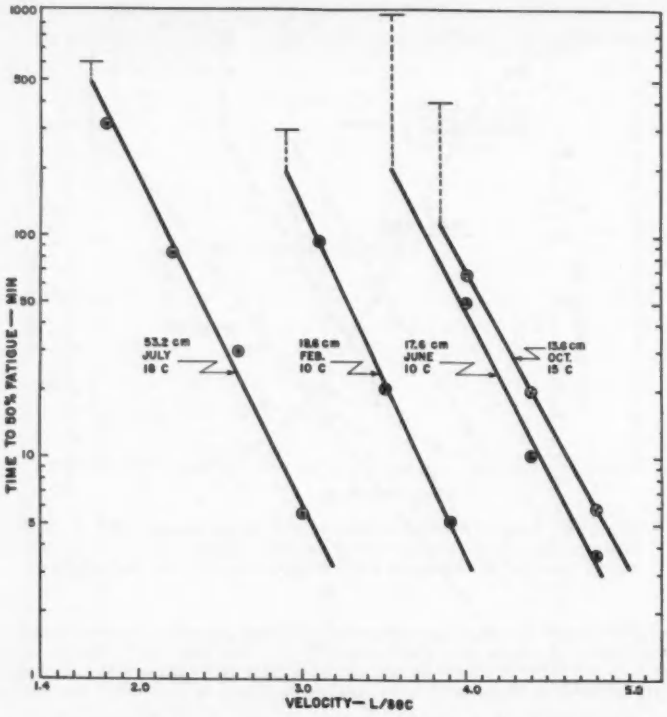

FIG. 3. The relation between time to 50% fatigue (FT_{50}) and velocity (length/sec) for a variety of sizes of sockeye salmon. The temperatures and times of year when the fatigue curves were obtained are indicated. Sustained performance of more than 50% of the fish occurred at the points of inflection (broken lines). Horizontal lines depict the termination time for each test. Experiments on adult salmon (53.2 cm) from Brett and Shoop (unpublished data); those for 18.6 cm and 17.6 cm yearling sockeye from Brett (1964).

As a result of an introductory velocity the fish always rated a minimum speed despite the fact that a sustained performance in a lethal zone is obviously impossible. For this reason the graph shows a baseline at the minimum applied velocity. Relatively little capacity and time to perform are present above 27.5 C.

DISCUSSION

FATIGUE TIME

The method of bioassay analysis can be seen to provide a useful way of expressing results of swimming speed tests. The problem of dealing with variability of response has been discussed by a number of authors. Bainbridge (1962) noted that gradual acceleration over a period of 10–15 min was required when introducing fish to higher velocities, but that "some specimens could not be induced to swim steadily at even slow speeds and a considerable degree

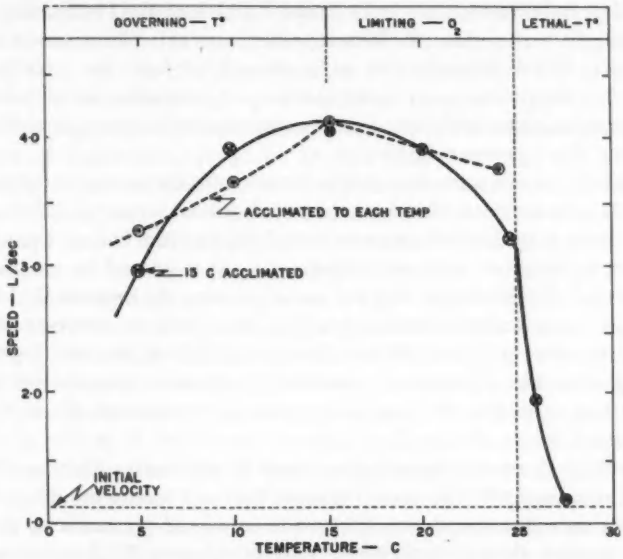

FIG. 4. The relation between swimming speed of sockeye (16.8 cm mean length) acclimated to 15 C only and tested at various temperatures, and of sockeye (17.8 cm mean length) acclimated to each temperature (from Brett, 1964). The relation of environmental factors of temperature and oxygen (air-saturated) on activity are indicated in the manner of Fry (1947).

TABLE II. The relation between temperature and the critical swimming speed of fingerling sockeye salmon acclimated to 15 C. Tests were conducted in November–December using five fish per test. Every 45 min the velocity was increased by 0.3 ft/sec (9.2 cm/sec) from an initial velocity of 0.7 ft/sec (21 cm/sec).

Temperature (C)	Total length (cm)		Wet weight (g)		Critical speed 45 min (L/sec)		60 min (L/sec)
	Mean	SD	Mean	SD	Mean	SD	Mean[a]
5.0	16.9	1.3	49.3	12.6	3.04	0.37	2.94
10.0	15.7	2.7	40.5	24.8	4.01	0.73	3.92
15.0	16.2	1.4	43.0	11.8	4.16	0.42	4.06
20.0	17.2	1.0	51.3	11.8	4.00	0.28	3.90
24.5	16.2	0.8	44.1	7.2	3.34	0.27	3.24
26.0	17.0	1.9	52.1	19.2	2.07	0.67	1.97
27.5	18.4	1.3	62.2	14.4	1.29	0.08	1.19

[a]Corrected to 60-min equivalent by subtracting 0.1 L/sec (see text).

of variability in behaviour might be found." Davis et al. (1963) using apparatus and techniques very similar to those reported here attempted to use the median performance (third fatigued fish of a sample of five) for juvenile chinook salmon (*O. tshawytscha*) and coho salmon (*O. kisutch*) but obtained highly variable results, apparently because of swimming advantage gained from small eddies near the upstream baffle.

Variability of the sort observed is not uncommon in time mortality curves. Bliss (1937) reported that truncated distributions occur in which individual response times may "depart more or less abruptly from the straight line which fits the remainder but analysis follows the same procedure as for complete distributions." No advantage was obtained by using the reciprocal of the fatigue time in relation to velocity as suggested in the bioassay analyses of Gaddum (1953), a transformation which would have facilitated determining the appropriate exposure time necessary to establish the maximum sustained speed. The minimum test time may be seen (Fig. 2 and 3) to occur at about 120 min for fingerling sockeye at 15 C.

A great many swimming tests have been conducted by Thomas et al. (1964) on fingerling salmon with a tunnel "devised for large scale evaluation of stamina in hatchery fish in a search for differences imparted by artificial propagation and not precise determinations of swimming speed." Increasing velocity increments were applied to obtain a performance index. While the need for such an approach is understandable, it is unfortunate that no swimming speeds are reported in a manner which would permit comparison with any of the literature on fish performance. It is apparent from their account, however, that similar problems of variability occur, and that these can be ascribed to one or other of such factors as temperature, turbulence, numbers, size, shape, diet, time from last feeding, muscular condition (pre-exercise), season, disease, and parasitism.

Temperature Effects on Performance

The combined results of swimming-speed tests on young sockeye in relation to temperature have been plotted in Fig. 4. The dome-shaped relation for the fish acclimated to 15 C within their zone of temperature tolerance is similar in general shape to the series of curves reported by Fry and Hart (1948) for cruising speeds of goldfish. By comparison the relation for fully acclimated yearling sockeye is quite different. A third shape appears in the lethal range.

Consideration of the relation governing these shapes will be made using the terms introduced by Fry (1947) in regard to the effects of environmental factors on animal activity.

The fully acclimated yearlings showed a steady increase in their capacity to perform up to 15 C. From work on the accompanying metabolic rates (Brett, 1964) it was apparent that availability of oxygen was not limiting performance at lower temperatures. Some aspect of internal control appeared to be operating as the limiting factor, possibly in the capacity to deliver oxygen (circulatory), remove metabolic products (excretory), provide adequate substrate, or activate

enzymatic processes (cellular). Since much higher but brief burst speeds could be achieved, energy liberation at the muscle site was not likely the failing mechanism. This may be considered a case of temperature acting in the role of a *governing factor* permitting higher sustained metabolic rates and therefore greater activity at higher temperatures. As stated, the relation changes at a temperature of approximately 15 C. In this region it has been shown that environmental oxygen availability within the limits of maximum gill ventilation has entered the picture — the supply of an external metabolite is acting as a *limiting factor*, but only at the highest levels of sustained performance.

In the case of the fingerlings acclimated to 15 C it may be noted that within ±5 C of the optimum performance (at 15 C) there is only a small reduction in swimming speed, demonstrating a relative independence of acclimation temperature in this range. At the extremes of 24.5 and 5 C it is apparent that temperature is exerting a restricting influence, particularly at low temperatures.

In the *zone of thermal resistance* where temperature enters as a *lethal factor* the inevitable effects of drastically lowered performance can be seen (Fig. 4). At 1 C degree above the *ultimate lethal temperature* (i.e. at 26 C) the 60 min sustained speed was about one-half the optimum. At 2.5 C degrees above the lethal level, performance had virtually reached the extinction point.

It can be concluded that a temperature of 15 C is physiologically ideal for young sockeye salmon in freshwater, a point which has been supported by evidence for optium growth in the presence of unrestricted ration (Donaldson and Foster, 1941). However, at 5 C, through acclimation, young sockeye can retain three-quarters of their capacity for optimum sustained performance, and, as has been postulated, they are probably thermally independent during the brief period of burst speeds (Brett, 1964).

Recommendations

It is most desirable that a set of standard procedures for the *fixed velocity* and *increasing velocity* techniques be established in order to facilitate comparisons among and between species. Although not necessarily employed where a particular index is sought, some additional tests could be conducted along set lines such that the index would have wider comparative value.

The time proposed for *fixed* velocity tests is a minimum of 200 min, following an introductory period at low velocity, sufficient to result in general quiescence among the test fish. It must be noted that excitement from handling can require a number of hours for recovery, a point which is well illustrated by the lengthy recovery times for fatigued fish, extending from about 3.5 hr (Brett, 1964) to 8–12 hr (Black, 1957; Black et al., 1962; Paulik et al., 1957). It is felt that the difference in level of applied velocity of 0.4 L/sec used in experiments on young sockeye was on the high side. A difference of 0.2–0.3 L/sec would have been better for defining the maximum sustained speed.

With regard to *increasing* velocity steps, a test time of 1 hr is desirable at each stage since unrealistic high levels of critical swimming speed could result from a series of short exposure times. This is also a reasonable duration for

obtaining sufficient reduction of oxygen content to allow accurate measures of metabolic rate in some of the relatively large volumes used in closed-circuit systems. The magnitude of the increment of increase poses a problem since sustained speed is proportional to a fractional power of length, of the order of $L^{0.6 \pm 0.1}$ (Bainbridge, 1962; Brett, 1965). It would appear best to resolve this problem by applying increments which are about one-eighth the maximum sustained speed, determined by preliminary experiments, otherwise an increment of something like 1.6 $L^{0.6}$/sec would be necessary.

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Mr D. B. Sutherland provided technical assistance and carried out many of the tests on swimming speeds of young salmon. Mr C. T. Shoop provided similar assistance in the work on adult salmon. It is a pleasure to acknowledge this excellent help and the cooperative spirit with which it was conducted.

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Movements of Brook Trout in Relation to an Artificial Pond on a Small Stream[1]

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ABSTRACT

A 20-year study showed that forming a pond near the mouth of Ellerslie Brook, Prince Edward Island, did not change the seasonal pattern of brook trout movements between fresh- and salt water. Delay in movements of trout through the pond and a tidal pool below the dam resulted in heavy natural and angling mortalities in those areas. Movements between fresh- and salt water were almost eliminated. Natural seeding of stream above pond was not demonstrably affected.

INTRODUCTION

A study has been in progress since 1946 at Ellerslie Brook, Prince Edward Island, to evaluate the effects of an artificial pond on movements, populations, and cropping by anglers of anadromous fish, chiefly brook trout (*Salvelinus fontinalis* (Mitchill)) and Atlantic salmon (*Salmo salar* Linnaeus). Effects of the pond on populations and movements of Atlantic salmon have been reported (Saunders, 1960). Movements of brook trout between and within fresh- and salt water (1946–52) before the pond was formed were described (Smith and Saunders, 1958). Movements of trout in the Ellerslie System (stream, pond, and estuary) after pond formation are described in this paper and are compared with movements observed in pre-pond years.

METHODS

A pond was formed to produce ecological changes on Ellerslie Brook. Additional installations were used to study the behaviour of the trout. These and other methods are given below as far as convenient in chronological order.

(1) A 2-way counting fence was operated year-round at the head of tide on the brook since June 1946 to evaluate movements of trout between the brook and its estuary. The two traps in the fence were cleared daily. The design and operation of this fence, designated estuarial fence, have been described (Smith and Saunders, 1956).

(2) In September 1950 a second fence, designated stream fence, was erected across the stream about 650 yards (594 m) above the estuarial fence to study the movements of trout into and out of the lower stream area that would later be flooded by the pond.

(3) During the summer of 1952, a dam was built across the mouth of the stream about 30 yards (27 m) above the estuarial fence. The 5-acre (2-ha) pond was formed by early November, and flooded the lower brook up to the stream counting fence (Fig. 1). During the spring of 1953, a large pool was scoured out below the dam. Pond and pool are described later.

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(4) Post-pond fence operations were similar to those in the pre-pond years. Trout descending through the stream fence entered the pond and were free to move through it, over the dam into the tidal pool, and on through the estuarial fence into the estuary.

(5) Trout ascending from the estuary were barred from moving beyond the tidal pool by the dam. To study movements of these trout in the pond, all incoming trout were removed from the upstream trap in the estuarial fence and placed in the pond from 1953 through 1955. To observe the possible effects of barring incoming sea-run trout on the population above the dam, none was placed in the pond after December 31, 1955; instead they were released in the tidal pool below the dam.

(6) During the study, trout older than age 0 (fish of the year) trapped at the fences, or captured by other means elsewhere in the system, were given a serially numbered jaw tag (Smith, 1957). To assess tag loss, the adipose fin of each trout was clipped at the fences; losses amounted to about 5%.

(7) An annual creel census was maintained on the Ellerslie System, 1947–65.

(8) An annual stream population census (electrofishing) provided information on the movements of tagged trout.

ELLERSLIE BROOK SYSTEM

Ellerslie Brook

Ellerslie Brook was described previously by Smith and Saunders (1958). It flows into Malpeque Bay on the north shore of Prince Edward Island. In summer the brook, including its tributaries, is about 6 miles (10 km) long, 4½–5 miles (7–8 km) of which support trout populations. The drainage area, underlain with Carboniferous sandstone, consists of about 10 miles^2 (26 km^2) of woodlots and farmland.

The water is moderately hard (*p*H 7.5–7.8) with a total hardness, as $CaCO_3$, of about 90 ppm, and a calcium content of about 30 ppm. Water temperature on the lower part of the stream exceeded 20 C (68 F) during the warmest summer days, but then fell into the teens overnight. When ice-covered, the temperature of the brook held consistently between 0 and 0.1 C (32 and 32.2 F).

Ellerslie Pond

The pond (5 acres; 2 ha) on the lower, in part tidal, reaches of Ellerslie Brook was filled in November 1952 (Fig. 1). The dam consists of four spillways, each 5 ft wide (1.5 m), and earthen embankments on either side. The water is held in the spillways with stop-logs. The stop-logs can be removed to drain the pond to original stream level. A spillway, to one side of the dam, was constructed through the retaining earth embankment as a safety measure to cope with unusual spates.

No water spilled over the dam when brook levels were seasonally low, for the most part in late summer and early autumn, and in mid-winter in the years 1959–63. Leakage through the dam structure, seepage through the lateral earth embankment, and evaporation were at times greater than inflow. The most protracted period (June 24–November 4) of no spill over the stop-logs was during the exceptionally dry summer and autumn of 1960. Usually a drop in

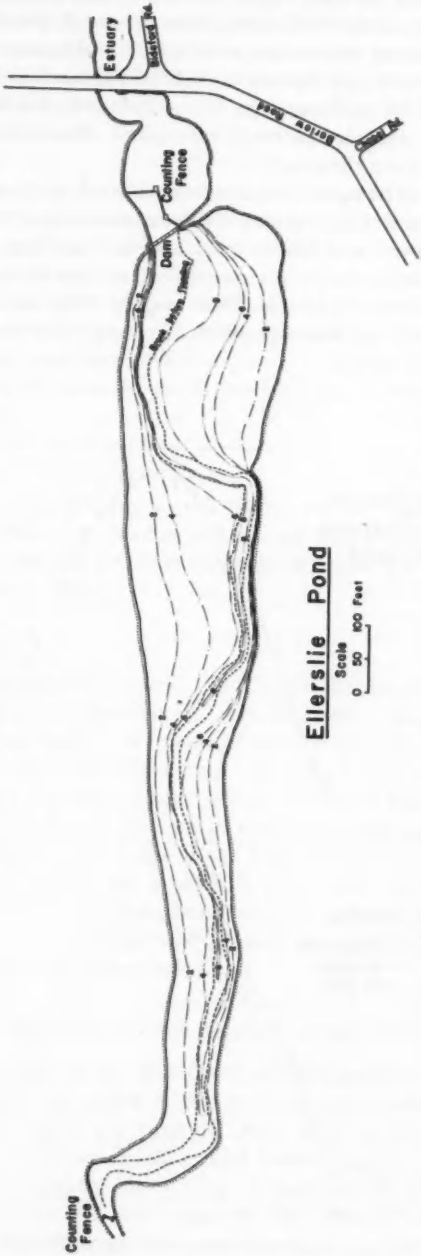

FIG. 1. Map of Ellerslie Pond from a survey by H. R. Found. Depth contours in feet.

pond level of over 1 ft (0.3 m) below the top of the stop-logs was not experienced for prolonged periods. Before and after the pond, few, if any, trout moved through the traps during low-water periods in late summer and early autumn, or in mid-winter (Smith and Saunders, 1958) (Tables I–IV). No spillage over the dam coincided with such periods. It is concluded that the physical barrier of the dam at low pond levels had little or no effect on naturally inclined movements of trout from the pond.

Monthly mean afternoon temperature of pond surface water from 1953 through 1963 is presented in Fig. 2A. Temperatures were taken daily between 4 and 5 PM, at the lower end of the pond, and for the most part represent the daily maxima. Monthly mean minimum and maximum temperatures of bottom water during the period September 1958 to July 1961 are shown in Fig. 2B. These records are from a thermograph at the dam, and readings are for near maximum depth of the pond.

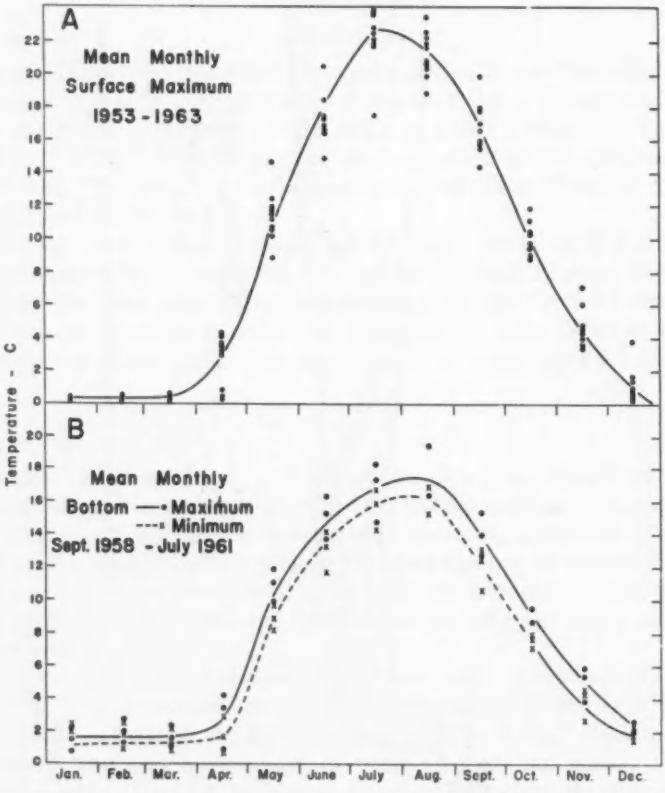

FIG. 2. Water temperature of the lower part of Ellerslie Pond.

Dissolved oxygen content of water in the pond at the surface (just under ice in winter), mid-depth, and bottom was determined at monthly intervals from January to August 1956. Oxygen content varied from 8.8 to 13.7 ppm and from 68 to 104% saturation. The lowest saturation value was encountered at the bottom on March 1, and the highest at the surface on August 9 when there was a moderate algal bloom. Range in *p*H values over the above period in 1956 was from 7.0 to 8.5.

Bottom fauna at 15 stations throughout the pond was sampled with an Eckman dredge on 15 occasions at all seasons from 1953 to 1958; this provided 225 samples. The number of organisms varied from a low of 33 to a high of 244 per ft^2 (355 to 2626 per m^2). Chironomid larvae predominated, with good representation of other insect larvae, snails, sphaeriids, and aquatic earthworms.

Fish occurring in the pond in addition to the brook trout were juvenile salmon (early years), eels, and sticklebacks.

In general, conditions in the pond, as shown above, were suitable for brook trout.

Pool Below Dam

Outflow from the pond dug a pool below the new dam during freshets in the spring of 1953. The pool is tidal and has an area of approximately 4000 ft^2 (370 m^2), with a maximum depth at low tide of about 5 ft (1.5 m) along much of the face of the dam apron.

Ellerslie Estuary

The geography and hydrography of Ellerslie Estuary have been discussed by Needler (1941) and Saunders and Smith (1964). Salinity measurements in the estuary approximately 300 yards (274 m) below the dam during autumn and early winter of 1961–63 ranged from 1 to 12‰ at the surface and 9 to 23‰ at the bottom (1 m). Salinities varied within the above ranges dependent upon phase of tide and volume of freshwater discharge from the brook. Values in general decreased toward the head of the estuary, and increased seaward into Malpeque Bay. When reference is made in this paper to trout moving to salt water (or estuary) it is implied that at least some entered water with salinities up to 29‰ as shown by their capture in smelt nets set in the lower part of the estuary in water reaching this salinity.

MOVEMENTS OF TROUT BEFORE POND FORMATION

Smith and Saunders (1958) illustrated and discussed movements of Ellerslie brook trout between and within fresh- and salt water before pond formation, 1948–52. Prominent upstream runs of trout were in April, June–July, and November, and downstream in May, and from October to early January. It was determined from tagging that during a specific run, some trout were delayed or remained in pools on the lower part of the brook between the fences and that others were recruited from this stream area.

MOVEMENTS OF TROUT AFTER POND FORMATION

Season of Movements

Recorded numbers of trout captured at the stream and estuarial fences for each month after the pond was formed in 1952 are given in Tables I–IV. The seasonal pattern of movements of trout was not affected by the pond (see Smith and Saunders, 1958). Movements continued to be largely in the spring and the autumn and early winter periods, which are seasons with water temperature rising and falling between winter low and summer high, with the highest water levels in the stream and pond, and with greatest changes in flow.

Prominent runs of trout on specific days before and after pond formation were associated with heavy rains and sharp increase in flow of water from the Ellerslie System.

Movements and Transfer of Trout into the Pond

The numbers of trout that were known to have run from the stream into the pond are given in Table I. The numbers include: (1) trout caught at the stream fence, tagged, and released into the pond; (2) trout tagged in the stream during annual population studies and later recaptured in pond or pool or at the fences without their movements from stream to pond having been detected; and (3) untagged individuals angled from the pond or captured at the stream fence as they moved from the pond. Some of the untagged trout in the pond in 1953 were those in the stream area that was flooded by the pond. Few such trout survived to be in the pond in 1954. Thereafter, untagged trout in the pond were those that ran undetected from the stream above. Some trout could have entered the pond undetected, there suffered natural mortalities, or moved from the pond without record of capture. Records in Tables I–IV show that the proportion of trout that escaped detection at the fences was at times large, presumably when there was malfunction of the fences with high water and with frazil ice formation. However, the chance of a trout making successive undetected movements past the fences is less likely (Smith and Saunders, 1956). When the pond was drained at the close of the angling seasons in 1958, 1962, and 1963, there were only 73, 19, and 29 trout remaining in the pond.

Incoming trout captured at the estuarial fence were tagged and transferred to the pond from 1953 through 1955 (Table V).

What Happened to Tagged Trout in the Pond

Records of what happened to tagged trout after entry into the pond are presented in Table V on a yearly basis. These records are supplemented in Table VI by showing what happened to tagged trout in certain prominent runs into the pond.

Movements from the pond.— From 1952–65, there were in the pond 8522 trout that had been tagged as they entered the pond past the stream fence (Table V). Of these, there are records of 2265 (27%) leaving the pond. The majority (1466; 65%) of those that left the pond continued downstream over the dam. However, 799 trout went no farther than the pond, and later returned to the stream.

TABLE I. Numbers of trout that moved from stream into pond as recorded by capture at fences and by angling. Numbers of undetected trout are given for years of capture; some moved undetected into pond in previous years.

	1952[a]	1953	1954	1955	1956	1957	1958	1959	1960	1961	1962	1963	1964	1965
Recorded at stream fence														
Jan.		86	0	6	31	0	0	0	14	0	0	82	0	0
Feb.		0	0	0	0	0	0	0	0	0	0	0	0	0
Mar.		0	0	0	0	0	0	0	0	0	0	0	0	0
Apr.		84	35	77	1	0	1	0	0	0	2	0	12	0
May		428	158	336	143	145	52	0	9	1	132	14	0	0
June		100	191	41	83	25	40	3	1	58	84	0	0	0
July		76	61	12	23	15	0	0	0	37	16	0	0	0
Aug.		34	0	6	0	0	22	15	0	0	0	0	0	0
Sept.		64	0	9	0	0	7	0	0	0	1	3	0	0
Oct.		110	244	9	0	0	116	0	0	41	1	0	0	0
Nov.	640	413	363	521	1537	189	25	64	45	56	61	6	46	44
Dec.	411	22	0	86	103	24	0	88	105	1	187	64	27	0
Total	1051	1417	1052	1103	1921	398	263	167	174	194	484	169	85	44
Tagged, undetected	0	364	103	3	182	142	40	87	65	5	80	177	138	47
Untagged, undetected	0	749[b]	791[b]	320	334	224	142	495	291	197	270	383	379	389
Annual total	1051	2530	1946	1426	2437	764	455	749	530	396	834	729	602	480

[a]Pond formed in early November 1952.

[b]Includes some trout that were in flooded area of stream.

TABLE II. Numbers of trout that moved from pond into stream as recorded by capture at stream fence, by angling, and by electrofishing in stream. Numbers of undetected trout are given for years of capture; some moved undetected into stream in previous years.

	1952	1953	1954	1955	1956	1957	1958	1959	1960	1961	1962	1963	1964	1965
Recorded at stream fence														
Jan.		0	0	0	0	0	0	0	0	0	0	0	0	0
Feb.		0	0	0	0	0	0	0	0	0	0	0	0	0
Mar.		0	0	0	0	0	0	0	0	0	0	0	0	0
Apr.		1	5	0	0	0	0	0	0	0	0	0	0	0
May		15	9	0	0	28	0	0	0	0	0	0	0	0
June		12	10	0	0	0	0	0	0	1	0	0	0	0
July		26	1	0	2	1	0	0	0	0	0	0	0	0
Aug.		1	0	0	0	0	0	0	0	0	0	0	0	0
Sept.		8	0	0	0	0	0	0	0	0	0	0	0	0
Oct.		10	73	2	0	0	0	0	0	0	0	1	0	0
Nov.	48	7	87	6	39	9	0	2	0	0	0	0	0	0
Dec.	6	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	54	80	171	8	41	38	0	2	0	1	0	1	0	0
Tagged, undetected	0	172	166	51	74	157	117	6	30	9	15	57	15	1
Annual total	54	252	337	59	115	195	117	8	30	10	15	58	15	1

TABLE III. Numbers of trout that moved into the estuary as recorded by capture at estuarial fence and by angling. Numbers of undetected trout are given for years of capture; some moved undetected into estuary in previous years.

	1952	1953	1954	1955	1956	1957	1958	1959	1960	1961	1962	1963	1964	1965
Recorded at estuarial fence														
Jan.		9	133	0	0	0	11	0	0	0	0	0	0	0
Feb.		4	0	0	0	0	0	0	0	0	0	0	0	0
Mar.		29	0	0	0	0	0	0	0	0	0	0	0	0
Apr.		53	237	11	0	0	27	27	12	0	0	0	0	0
May		158	176	1	15	100	3	7	2	20	0	0	3	2
June		104	59	3	5	2	10	17	2	0	1	0	1	0
July		22	1	0	0	0	0	1	0	0	0	0	0	0
Aug.		5	0	0	0	0	0	0	0	0	0	0	0	0
Sept.		5	0	0	0	0	0	0	0	0	0	0	0	0
Oct.	1	73	33	0	0	0	136	0	1	6	0	0	0	0
Nov.	2	222	43	12	114	64	46	98	5	2	3	0	0	0
Dec.	0	112	77	2	15	146	4	49	12	0	1	0	0	0
Total	3	796	759	29	149	312	237	199	34	28	5	0	4	2
Tagged, undetected	0	65	206	70	186	80	63	54	9	16	13	10	0	5
Annual total	3	861	965	99	335	392	300	253	43	44	18	10	4	7

TABLE IV. Numbers of trout that moved from estuary into pool area below dam as shown by capture at the estuarial fence, by angling, and by electrofishing in pool. Numbers of undetected trout are given for years of capture; some moved undetected into pool in previous years.

	1952	1953	1954	1955	1956	1957	1958	1959	1960	1961	1962	1963	1964	1965
Recorded at estuarial fence														
Jan.		0	0	0	0	5	0	0	0	0	0	1	0	0
Feb.		0	0	0	0	0	0	0	0	0	0	0	0	0
Mar.		0	0	0	0	0	0	0	0	0	0	0	0	0
Apr.		38	127	160	0	0	94	88	7	0	0	0	0	0
May		19	192	21	99	16	33	11	12	34	1	9	1	2
June		111	578	83	147	6	49	26	2	0	6	6	0	0
July		72	31	1	3	18	0	19	0	0	3	3	0	0
Aug.		21	1	0	1	3	0	0	4	0	0	0	0	0
Sept.		9	3	0	0	0	1	0	2	0	0	0	0	0
Oct.		57	12	0	0	1	86	9	0	0	6	1	0	0
Nov.	0	48	38	3	38	28	41	14	6	9	15	0	0	1
Dec.	0	7	2	1	3	13	0	6	2	0	2	1	0	0
Total	0	382[a]	984[a]	269[a]	291	90	304	173	35	43	33	21	1	3
Tagged, undetected	0	60	75	0	1	117	15	37	39	11	0	3	8	0
Annual total	0	442	1059	269	292	207	319	210	74	54	33	24	9	3

[a]Transferred to pond.

TABLE V. What happened to tagged trout after entering Ellerslie Pond, as shown by trapping and angling. Percentages of annual total in parentheses.

	No. into pond	Subsequently moved upstream	Subsequently moved downstream over dam	Number angled in pond: Within 1 month	Between 1 and 2 months	Between 2 and 3 months	After 3 months	Total angled	No. with no further record of recapture
(A) Trout that moved from stream into pond									
1952 (Nov.–Dec.)	1051	48 (5)	127 (12)	0	0	0	533	533 (51)	343 (32)
1953	1417	160 (11)	272 (19)	115	20	20	358	513 (36)	472 (33)
1954	1052	178 (17)	117 (11)	215	35	7	167	424 (40)	333 (32)
1955	1103	71 (6)	195 (18)	235	35	14	191	475 (43)	362 (33)
1956	1921	176 (9)	544 (28)	114	24	6	201	345 (18)	856 (45)
1957	398	27 (7)	46 (12)	50	16	10	65	141 (35)	184 (46)
1958	263	10 (4)	72 (27)	33	12	16	53	114 (43)	77 (26)
1959	167	21 (13)	18 (11)	5	4	1	54	64 (38)	64 (38)
1960	174	16 (9)	12 (7)	4	0	0	56	60 (35)	86 (49)
1961	194	20 (10)	16 (8)	32	6	2	29	69 (36)	89 (46)
1962	484	42 (9)	44 (9)	115	21	11	106	253 (52)	145 (30)
1963	169	15 (9)	3 (2)	5	0	1	83	89 (53)	62 (36)
1964	85[a]	14 (16)	0	2	1	1	19	23 (27)	48 (57)
1965	44[a]	1 (2)	0	0	0	0	2	2 (5)	41 (93)
Totals, 1952–65	8522	799 (9)	1466 (17)	925	174	89	1917	3105 (37)	3162 (37)
(B) Trout that moved from estuary into stream									
1953	381	34 (9)	89 (23)	75	16	12	38	141 (37)	117 (31)
1954	975	89 (9)	68 (7)	563	56	25	21	665 (68)	153 (16)
1955	262	24 (9)	15 (6)	117	29	7	3	156 (60)	67 (25)
Totals, 1953–55	1618	147 (9)	172 (11)	755	101	44	62	962 (59)	337 (21)

[a]Further records for some of these trout may be obtained in 1966.

TABLE VI. What happened to tagged trout after entering pond during specific runs. Percentage of total number entering pond given in parentheses.

Origin of movement:	From stream						From estuary	
Period of entry:	Nov. 1, 1952–Jan. 16, 1953		April 23–June 4, 1955		Nov. 10–30, 1956		April 1–June 30, 1954	
No. of trout	1132		462		1537		901	
Angled in pond	583 (52)		294 (64)		174 (11)		662 (74)	
Moved down over dam	141 (12)		34 (7)		502 (33)		55 (6)	
During same season	7		28		98		53	
During following year	134		6		404		2	
Angled in pool	6		25		327		13	
Moved into estuary	135		9		175		42	
Moved upstream	53 (5)		13 (3)		157 (10)		65 (7)	
During same season	13		13		35		65	
During following year	40		0		122		0	
No further record of recapture	355 (31)		121 (26)		704 (46)		119 (13)	

Among the 1618 trout that were transferred from the estuarial traps (1953–55), 147 moved through the pond into the stream, but a somewhat larger number, 172, went no farther than the pond and dropped back over the dam (Table V).

In summary, a minority (25%) of the trout tagged upon entry into the pond was later recorded as leaving it. Apparently, out-of-pond movements were sufficiently delayed to result in heavy natural and angling mortalities in the pond.

Out-of-pond movements, and any delay in these, were doubtless determined by a number of factors. The large living space in the pond was an important factor. We observed that movements of trout seaward before pond formation were often delayed or terminated in the larger pools on the lower reaches of Ellerslie Brook (Smith and Saunders, 1958). We also found that an increased number of trout could be held in upstream, much smaller sections of Ellerslie Brook by altering the environment to provide small pools (Saunders and Smith, 1962). More recent and more extensive pool and cover improvement on 1800 yards (1646 m) of Ellerslie Brook above the pond resulted in higher standing crops of yearling (age I) and older trout (Fig. 3). The pond interposed a much

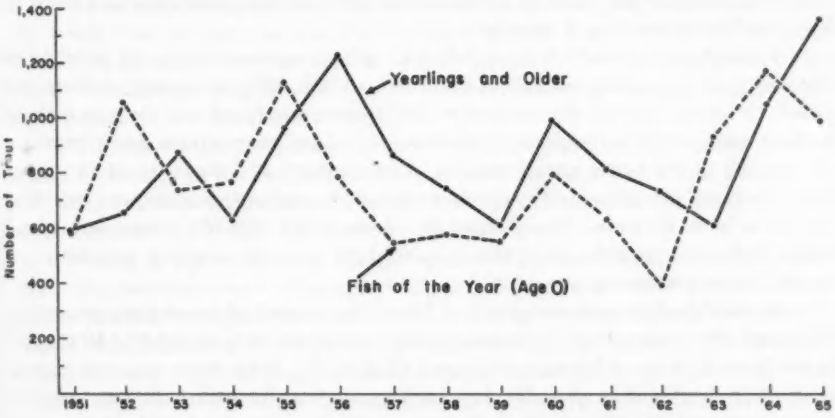

FIG. 3. Number of brook trout in 14 50-yard sections of Ellerslie Brook.

more spacious habitat between stream and estuary. Not only was more living space provided, but also a body of water in which there was relatively little current. Any stimulus and directive influence of current on the movement of trout in the stream were much diminished or lost when the fish entered the pond. Elson (1939) found that brook trout, under controlled conditions, wandered randomly when placed in still water after having become adapted to current. Trout may have moved randomly, at least for a time, after entering Ellerslie Pond. Trout that moved out of the pond usually did so in the spring

and autumn, or early winter, when change in flow of water was greatest in the stream and pond. Even then, especially in autumn and early winter, many trout did not pass through the pond, but remained there to be angled from the pond some months later (Table V).

Both pre- and post-pond movements of trout were affected by the low water temperature of winter. Before pond formation there was little movement of trout past the counting fences when water temperature fell to near 0 C (Smith and Saunders, 1958). Similarly, only a small proportion (6%) of the tagged trout that moved into the pond, November 1, 1952 to January 16, 1953, and November 10–30, 1956, moved out of the pond during the same autumn and early winter period in which they entered (Table VI). These were periods when water temperature was falling to or had reached winter low levels (Fig. 2).

Almost all movements of trout into the traps at the fences were nocturnal, both before and after pond formation.

Angling in the pond.— Delay in out-of-pond movements resulted in considerable exploitation of the trout in the pond by anglers. Of the known number (8522) of tagged trout that entered the pond from the stream, 3105 (37%) were angled from the pond (Table V). Of these trout, about 30% were angled within 1 month of having entered. However, tagging showed that the majority entered the pond in autumn and early winter, and thus had been in the pond for more than 3 months.

Table V shows that 1618 tagged trout were transferred into the pond from the estuarial fence. Over a half of these trout (962; 59%) were angled from the pond. Of those angled, the majority (78%) were captured within a month of having been put into the pond. The short period between release and recapture by angling of the latter group resulted from transfer of a majority of the trout into the pond in spring and early summer when angling pressure was heaviest for the season. However, it is known that some trout (62; 6%) remained in the pond beyond 3 months and were angled late in same angling season or in smaller numbers during the next.

No record after entry into pond.— Many of the tagged trout disappeared in the pond after entry. Such individuals (3499) made up 34% of the 10,140 tagged trout known to have been in the pond (Table V). They were not recaptured even though this was possible by angling, at two counting fences, and in population assessments made in the stream above the pond and in the pool below the dam. Doubtless some passed the two fences undetected and disappeared outside the pond area itself. However, a good proportion of the tagged trout not accounted for probably suffered natural mortalities in the pond. Saunders (1960) also showed in the early years of the pond that salmon smolts suffered heavy mortalities when delayed in the pond.

The trout were subject to predation while in the pond. Fish-eating birds, notably the kingfisher and great blue heron, frequent the Ellerslie System. Another predator of trout is the mink which are numerous on the system. In the period 1952–65, mink entered the fish traps and killed at least 253 trout; others were probably eaten or carried away from the traps and cached. Another

known predator was the American eel, which was more numerous and of larger size in the pond than in the stream before flooding (Smith and Saunders, 1954; Saunders, 1960).

Movement of Trout in Relation to Tidal Pool below Dam

At irregular intervals, assessments of the trout population in the pool were made over the period 1953–61, for the most part in summer and autumn. Assessments were made by electrofishing which was possible only at low tide and after pond overflow had flushed saline water from the pool. When captured, trout were tagged if they were not already bearing a tag. Records of the number of trout involved in the assessments and data on what happened to them are given in Table VII.

From 1953 through 1961 anglers caught 34% of the trout that were captured and released in the pool. Of the 241 trout captured and released in 1955, 90 were angled the following year from the pool. As was the case in the pond, delay in this large pool was apparently sufficient for considerable angling mortality to occur.

Movements between Fresh- and Salt Water

The recorded movements of trout from the stream into the estuary before the pond was formed varied, over a 5-year period, from 999 (1951) to 3104 (1949), at an annual average of 1735. Table III shows that the recorded number of movements into the estuary declined after pond formation, so that by 1960 and thereafter few trout were involved.

With the decline in number of movements of trout into the estuary, there was a concomitant decline in movements from the estuary (Table IV).

It is concluded that pond formation almost eliminated the movements of trout between fresh- and salt water at Ellerslie Brook.

As seen in Tables III and IV, and as shown by Smith and Saunders (1956), trout escaped detection as they moved past the estuarial fence both after and before pond formation. However, there is strong supporting evidence for the above conclusion. In addition to the capture of fewer trout at the estuarial fence, there was poorer angling success in the estuary, fewer trout in the pool below the dam, and a much decreased number of trout to be observed in pools below the estuarial fence with advance of years from pond formation.

There was no evidence that building a dam changed flow patterns of water to result in insufficient stimulus to move trout into Ellerslie Brook. Saunders and Smith (1964) found that hatchery-reared brook trout, planted in the estuary in 1961 and subsequent years, moved to the head of the estuary in response to season and water conditions as had been observed for naturally reared Ellerslie trout.

Movements in Relation to the Stream above the Pond

Mortalities among the trout while in the pond, and movements over the dam without possibility of later return, were circumstances which reduced the

TABLE VII. Records of what happened to trout after capture in population assessments in pool below dam. Percentage recapture of trout taken in assessments in parentheses.

Year of assessment	No. of assessments	No. of trout	Subsequent recoveries				No. further record
			Recaptured in pool with no further record	Angled from pool	Recaptured in estuarial traps	Angled[a] in pond	
1953	4	105	13 (12)	2 (2)	34 (32)	32 (31)	24 (23)
1954	3	212	30 (14)	12 (6)	74 (35)	68 (32)	28 (13)
1955	4	241	22 (9)	90 (37)	24 (10)	2 (1)	103 (43)
1956	1	28	4 (14)	14 (50)	2 (7)	1 (4)	7 (25)
1957	3	75	17 (23)	41 (54)	9 (12)	0	8 (1)
1958	1	24	0	9 (37)	9 (37)	0	6 (26)
1959	1	53	0	37 (71)	6 (11)	0	10 (19)
1960	1	3	0	2 (67)	0	0	1 (33)
1961	1	42	1 (2)	24 (57)	3 (7)	0	14 (33)

[a]Transferred to pond from estuarial fence.

runs of trout from the pond up into the stream. Consolidating the records for the period 1952–65, only about 9% of the trout tagged upon entry into the pond were later recorded as moving back into the stream (Table V). The percentage varied annually, being greatest in 1954 (17%) and 1964 (16%) without any consistent trend over the years.

Before the pond was formed, from 1950 when the stream fence was first put into operation until 1952 when the pond was filled, 2472 trout were recorded passing the stream fence into the stream area between the two fences (Smith and Saunders, 1958). Some of these fish ran farther into the estuary. Of the 2472 trout, 431 (17%) were again recorded as they returned upstream past the stream fence. Thus, before the pond, only a minority of the trout that left the stream above the level of the stream fence returned. However, the proportion that returned to the stream was less after pond formation. Accordingly, a direct effect of pond formation was to reduce the number of trout in the stream by the number that would have returned if the pond had not been there. The effect was moderate, however, even when the numbers of trout in the runs were greatest.

The standing crops of trout in fourteen 50-yard (45-m) sections of the stream above the pond were annually assessed in late summer and early autumn by electrofishing during the period 1951–65 (Saunders and Smith, 1954). The total numbers of trout in the assessment sections for each year are plotted in Fig. 3. These data have been considered representative of the annual changes in the population of trout in the stream. The numbers of yearling and older trout in the annual standing crops varied as much as 100% — 1230 in 1956 and 614 in 1959 — without any consistent trend over the years that might be attributed to pond formation. The annual standing crops of fish of the year (0+) also varied widely and were lower from 1957 to 1962 than previously or later. The variations and level of standing crops of both fish of the year and older trout were more associated with changes in the brook environment than with any demonstrable effect of pond formation. The silt load, for instance, was shown to have a direct deleterious effect (Saunders and Smith, 1965). A definite upswing in the standing crops in 1963–65 has been attributed to the previously mentioned stream improvement carried out in 1962. A reduced return of trout to the stream that appeared to result from pond formation did not jeopardize the seeding of the stream. (A full account of annual variations of the standing crops of trout in Ellerslie Brook and the relation of environmental changes to these is in preparation.)

SUMMARY

1. A 5-acre pond was formed in November 1952 just above the head of tide on the lower dart of Ellerslie Brook, Prince Edward Island. Limnological conditions in the pond are described.

2. The movements of trout after pond formation were contrasted with those observed lover a 6-year period by similar means before the pond was formed.

3. Movements of brook trout within the Ellerslie System (stream, pond, and estuary) after pond formation were followed by capture, tagging, and recapture at a counting fence in

the estuary at the head of tide and another in the stream just above the pond, as well as during annual assessments of standing crops of trout in the stream. Trout were also captured during assessments of standing crops of trout in the pool below the dam, when the pond was drained in certain years, and by anglers (annual creel census).

4. Seasonal patterns of movements past the fences were unchanged after pond formation. The runs continued to be dominantly in spring, autumn, and early winter, seasons when water levels and temperatures were subject to greatest changes.

5. Ascending trout transferred from the estuarial fence into the pond, and those that descended from the stream, were delayed in the pond for varying periods of time. Delay in out-of-pond movements was associated with a relatively large living space and a reduced velocity of water in the pond. About 25% of the trout that entered the pond later left it. About 37% of the trout that left the pond reversed the direction of movement after entry into the pond, either to run back into the estuary or into the stream above. Comparable was the pre-pond observation that trout reversed direction after entering the stream area between the fences, with its relatively large pools.

6. Delay in out-of-pond movements was sufficient to result in heavy combined natural and angling mortalities in the pond. The average annual exploitation rate by anglers of the tagged trout in the pond was about 40%. Many tagged trout (35%) disappeared without further records of capture after entry into the pond. Some of the loss may be attributed to undetected movements past the counting fences and other to predation in the pond by fish-eating birds, mink, and eels.

7. The numbers of trout that moved from fresh- to salt water declined from an annual pre-pond average of 1735 to less than 100 with advance of years after pond formation. There was a concomitant decline in the numbers of trout ascending into the stream from the estuary. Pond formation on Ellerslie Brook resulted in the near elimination of runs of trout between fresh- and salt water.

8. The pond effected minor changes in the standing crops of trout in the stream above the pond. About 9% of the trout that ran from the stream into the pond returned to the stream. During the pre-pond period about 17% of the trout that ran into the stream area later flooded by the pond returned upstream. The loss thereby occasioned by the pond formation did not result in demonstrable reduction in the seeding of the stream.

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Aporocotyle margolisi n.sp. (Digenea: Aporocotylidae) from *Merluccius productus*[1]

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ABSTRACT

Aporocotyle margolisi n.sp., from the bulbus arteriosus of *Merluccius productus* (Ayres) caught off Vancouver Island, is described and figured. Records of *Aporocotyle* from other hosts are discussed.

INTRODUCTION

Three species have been assigned to the genus *Aporocotyle*, namely, *A. simplex* Odhner, 1900, from *Pleuronectes* (=*Limanda*) *limanda*, *Pleuronectes* (=*Platichthys*) *flesus*, and *Drepanopsetta* (=*Hippoglossoides*) *platessoides* off Kristineberg, Sweden; *A. orientalis* Yamaguti, 1934, from a *Cottunculus* sp. in Toyama Bay, Japan; and *A. spinosicanalis* Williams, 1958, from *Merluccius merluccius* in the European North Atlantic. *Aporocotyle odhneri* Layman, 1930, was transferred by McIntosh (1934) to *Paradeontacyclix* by reason of its short post-ovarial uterus and post-ovarial genital pore.

So far as is known, adult *A. spinosicanalis* are restricted to the heart and bulbus arteriosus of *M. merluccius* (Smith and Williams, 1967). Accordingly, hearts preserved in 4% formaldehyde and otoliths from 26 specimens of the Pacific hake, *M. productus* (Ayres), were received from the Fisheries Research Board of Canada, Biological Station, Nanaimo, B.C. These fish were caught by the research vessel *G. B. Reed* in March 1965 at the general locality shown in Fig. 1. Data relating to the fish are given in Table I.

Examination of the hearts by the method described by Smith and Williams (1967) revealed four specimens of an aporocotylid in three hearts (No. 2, 11, and 13 in Table I). All four flukes were found in the bulbus arteriosus. Whole mounts were prepared of three flukes, two of which were stained in Ehrlich's haematoxylin and one in Celestin blue and Chromotrope 2R. The remaining specimen was sectioned in a longitudinal coronal plane at 5 μ and treated with Mallory's triple stain.

Since the specimens do not agree with the original accounts of *A. simplex* Odhner, 1900, *A. orientalis* Yamaguti, 1934, or *A. spinosicanalis* Williams, 1958, or with the many specimens of the last-named species examined during recent studies, they are assigned to a new species, *A. margolisi* n.sp. In the following description the measurements and other features of the holotype are given first with those of the two paratypes in parentheses.

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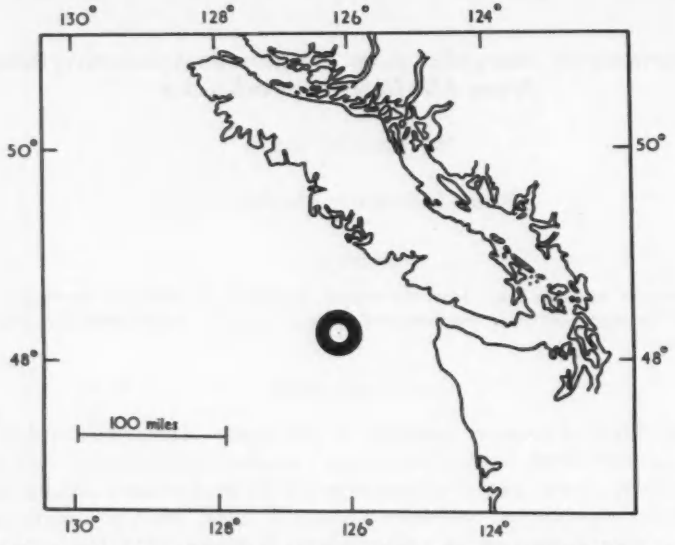

FIG. 1. Locality off Vancouver Island where 26 specimens of *Merluccius productus* were collected.

Aporocotyle margolisi n.sp.

EXTERNAL FEATURES, INTEGUMENT, AND MUSCULATURE

The specimens are greyish white and measure 4.96 mm (1st paratype, 3.82 mm; 2nd paratype, 4.86 mm) long, and 1.39 mm (1st paratype, 1.14 mm; 2nd paratype, 1.14 mm) in maximum breadth. The body (Fig. 2) is broadest at the middle and tapers anteriorly and posteriorly to lanceolate extremities.

The ventral surface, lateral margins, and dorsolateral areas of the body bear groups of spines (Fig. 4); thus, the greater part of the dorsal body surface is free from spines. The spines are about 0.017 mm long and arranged in groups of 20–40; the distance between each group is about 0.022 mm (Fig. 5). The cuticle is thin. Although a detailed study of the various muscle layers in the body wall was not possible, the horizontal sections show evidence of delicate circular and longitudinal muscles (Fig. 3). There are no suckers. Host red blood cells are often found closely adhering to the outer surface of the body (Fig. 3).

ALIMENTARY CANAL, EXCRETORY AND NERVOUS SYSTEMS

The mouth is terminal and leads into the oesophagus which measures 1.43 mm (1st paratype, 1.07 mm; 2nd paratype, 1.27 mm) and about 0.07 mm in diameter. A short anterior portion of the oesophagus appears to be lined with cuticle; the remainder is lined by flagellae approximately 0.017 mm long (Fig. 7). The anterior two-thirds of the oesophagus is surrounded by numerous

TABLE I. Data on the 26 specimens of *M. productus* examined for *Aporocotyle*.

Serial No.	Length (*cm*)	Sex	Age (*years*)	Locality[a] N lat	Locality[a] W long	Depth (*fath*)	Date in 1965	No. of flukes found
1	54	♀	7+	48°18′	126°05′	238–224	March 13	–
2	54	♂	7+	48°19′	126°06′			2
3	55	♀	9+					–
4	55	♀	8+					–
5	57	♀	9+					–
6	58	♀	11+					–
7	58	♀	11+					–
8	63	♀	13+					–
9	63	♀	9+					–
10	66	♀	11+					–
11	47	♀	5+	48°17′	126°03′	264–246	March 13	1
12	55	♀	6+	48°18′	126°04′			–
13	58	♂	7+					1
14	58	♀	9+					–
15	59	♀	12+					–
16	55	♀	9+	48°20′	126°07′	276–312	March 13	–
17	56	♀	10+	48°19′	126°06′			–
18	57	♀	8+					–
19	67	♂	12+					–
20	67	♂	12+					–
21	69	♂	12+					–
22	72	♀	12+					–
23	63	♂	10+	48°18′ 48°19′	126°03′ 126°04′	188–188	March 14	–
24	54	♀	9+	48°46′	126°34′	226–262	March 17	–
25	58	♀	9+	48°45′	126°32′			
26	66	♂	8+					–

[a]The first set of coordinates indicates the position when the trawl was shot, the second set the position when it was hauled. Depth is shown in the same manner.

small gland cells, whereas the posterior one-third is surrounded by larger and elongate gland cells (Fig. 2). A small sphincter muscle is present at the junction of the oesophagus with the transverse limb of the H-shaped intestine (Fig. 3).

The posterior limbs of the intestine terminate near to the end of the body, the left limb being somewhat shorter than the right, this feature being particularly pronounced in one of the paratypes. The anterior limbs of the intestine are about one-third the length of the posterior (Fig. 2).

The contents of the gut are interesting for the following reasons. In the longitudinal coronal sections the oesophagus contains erythrocytes (Fig. 7) corresponding in size and structure to those which are often closely adherent to the outer surface of the body (Fig. 3). The intestine of the sectioned specimen contains bodies of variable size (Fig. 8) whereas the hindmost parts of the posterior limbs show elongate structures like those in the whole mount stained

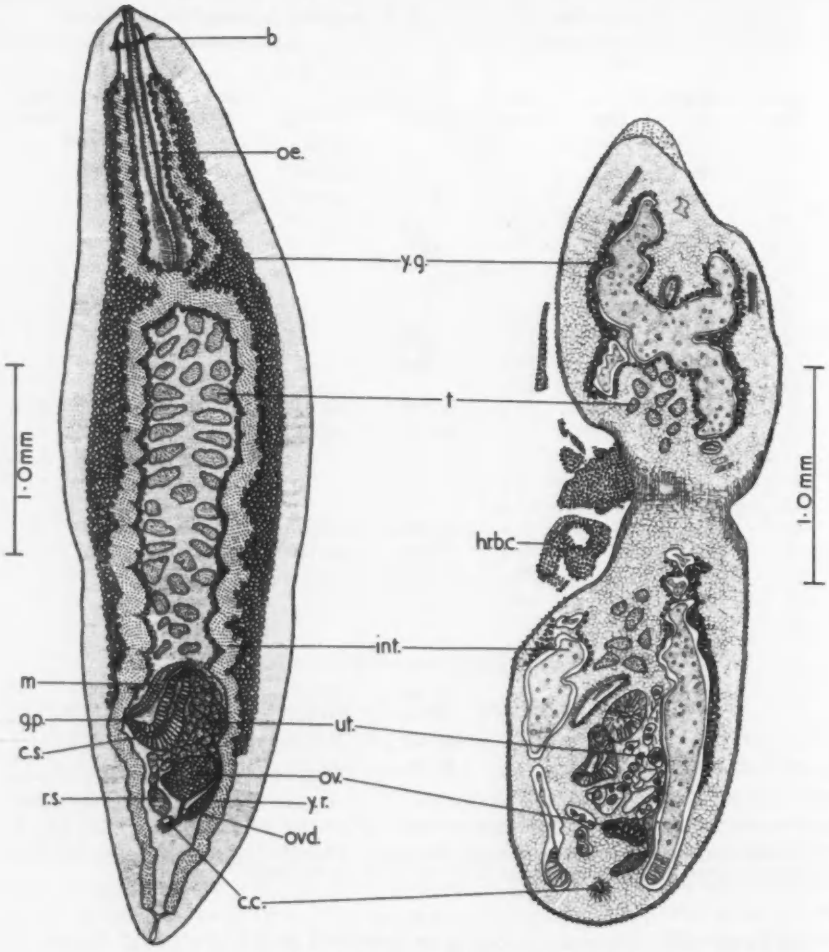

FIG. 2. (*Left*). *Aporocotyle margolisi* n.sp., dorsal view.

FIG. 3. (*Right*). *Aporocotyle margolisi* n.sp., longitudinal coronal section. KEY TO ABBREVIATIONS: *b* = brain; *c.c.* = central chamber; *c.s.* = cirrus sac; *g.p.* = genital pore; *h.r.b.c.* = host red blood cells; *int.* = intestine; *m* = metraterm; *oe.* = oesophagus; *ov.* = ovary; *ovd.* = oviduct; *r.s.* = receptaculum seminis; *t* = testes; *ut.* = uterus; *y.d.* = yolk duct; *y.g.* = yolk gland; *y.r.* = yolk reservoir.

with Celestin blue and Chromotrope 2R (Fig. 6). The latter structures are about 0.043 to 0.065 mm long and 0.007 to 0.011 mm broad. They took up the Orange G from Mallory's triple stain and the Chromotrope 2R from the Celestin blue–Chromotrope 2R mixture. According to Gurr (1965) both these stains are sulphonated wholly anionic dyes with –OH groups; they also have similar molecular weights (Chromotrope 2R, 468; Orange G, 452).

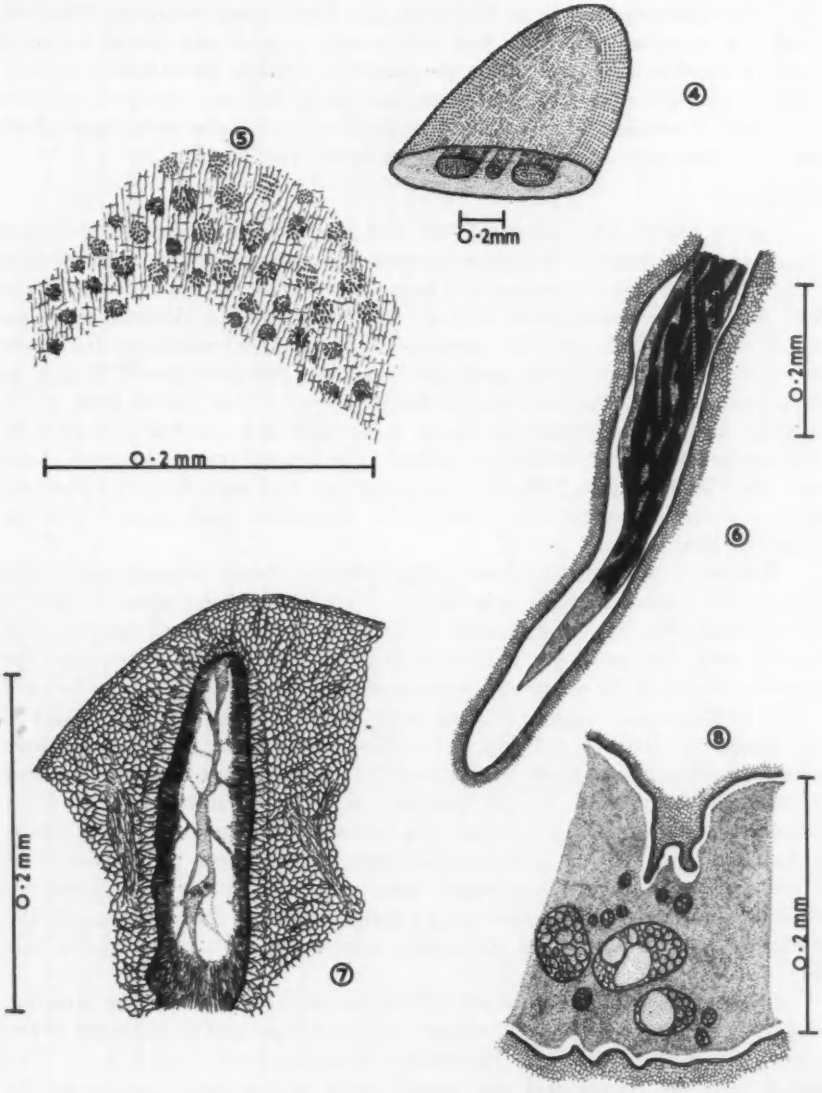

FIG. 4. Stereodiagram showing distribution of spines on *A. margolisi.*

FIG. 5. Facial view of spines on *A. margolisi.*

FIG. 6. Right posterior intestinal caecum showing contents.

FIG. 7. Oesophagus showing flagellae.

FIG. 8. Intestine at junction with oesophagus showing inclusions.

The excretory vesicle is Y-shaped, the main stem extending anteriorly from the terminal pore and then bifurcating to give two lateral collecting tubules which run forward under the posterior limbs of the intestine (Fig. 2). The "brain" lies dorsal to the oesophagus about 0.16 mm from the anterior extremity; it consists of two ganglia, joined by a transverse commissure, from each of which arise anterior, lateral, and posterior nerves (Fig. 2).

Genitalia

There are 35 (1st paratype, 46; 2nd paratype, 42) irregularly shaped testes which fill much of the space between the posterior limbs of the intestine (Fig. 2, 3). The vasa efferentia unite near the posterior margin of the testicular field to form the vas deferens (Fig. 2); this opens into the elongate cirrus sac which measures 0.68 mm (1st paratype, 0.59 mm; 2nd paratype, 0.51 mm) in length and 0.27 mm (1st paratype, 0.31 mm; 2nd paratype, 0.20 mm) in maximum breadth. The cirrus sac is slightly constricted in two or three places (Fig. 2). The vesicula seminalis interna is surrounded by prostatic gland cells. The cirrus opens into the genital atrium. The genital pore is situated about 1.39 mm (1st paratype, 1.00 mm; 2nd paratype, 1.23 mm) from the posterior extremity and about halfway between the left lateral body margin and the dorsal midline (Fig. 2).

The ovary is roughly oval with slight lobation, lying obliquely just to the right of the midline with its right margin touching the right posterior limb of the intestine (Fig. 2). It measures about 0.29 by 0.18 mm (1st paratype, 0.28 by 0.15 mm; 2nd paratype, 0.27 by 0.20 mm). The oviduct arises from the posterior border of the ovary and, having received the yolk duct, turns forward in the midline and becomes swollen to form the central chamber which is surrounded by Mehlis' gland (Fig. 2, 3). The proximal part of the uterus forms a distinct receptaculum seminis uterinum (Fig. 2) which then continues forward between the ovary and the left posterior limb of the intestine. The much coiled ascending limb of the uterus lies between the ovary and testes and is packed with eggs (Fig. 2). The uterus changes its course near the proximal end of the cirrus sac and turns obliquely backwards as the relatively short descending limb which opens into the genital atrium; the distal portion of the uterus is modified to form a metraterm surrounded by distinct gland cells (Fig. 2, 3).

The vitellaria consist of small follicles around the limbs of the intestine (Fig. 2, 3). A very prominent yolk reservoir lies behind and to the right of the ovary and communicates with the oviduct by a short yolk duct (Fig. 2). The uterine eggs are thin-shelled and ovoid, those in the distal portion of the ascending limb of the uterus measuring about 0.043 by 0.022 mm. Their structure is similar to that described by Williams (1958) for the eggs of *A. spinosicanalis*.

DISCUSSION

The differences between the four species of *Aporocotyle* are shown in Table II. *Aporocotyle margolisi* n.sp. differs from the other three species

TABLE II. Differences between the four species of the genus *Aporocotyle*.

Species:	*A. margolisi* n.sp.	*A. simplex*	*A. orientalis*	*A. spinosicanalis*
Host(s):	*Merluccius productus*	*Pleuronectes* (= *Limanda*) *limanda* *Pleuronectes* (= *Platichthys*) *flesus* *Drepanopsetta* (= *Hippoglossoides*) *platessoides*	*Cottunculus* sp.	*Merluccius merluccius*
Locality:	Off Vancouver Island, B.C.	Off Kristineberg, Sweden	Toyama Bay, Japan	European North Atlantic
Habitat:	Bulbus arteriosus	Gills and blood vessels	Heart and branchial vessels	Auricle, ventricle, bulbus arteriosus
Shape:	Elongate, extremities lanceolate	Elongate, flattened, anterior extremity pointed, posterior extremity rounded	"Flat, spindle shaped with more or less pointed extremities"	Elongate, extremities almost rounded; margins of body curved ventrally to form a canal
Length:	3.82–4.96 mm	3.50–5.00 mm	5.20–7.60 mm	3.96–6.30 mm
Breadth:	1.14–1.39 mm	0.45–0.75 mm	0.80–1.18 mm	0.86–1.17 mm
Spines:	0.017 mm long, in groups, absent from greater part of dorsal body surface	0.006 mm long, in groups, particularly well developed on ventral body surface	Body "entirely covered with minutes spines tending to form groups"	0.017 mm long, in groups, restricted to ventral canal
Ratio of oesophagus length to body length:	ca. 1:3.5	ca. 1:4.3	ca. 1:5.7	ca. 1:2.7
Number of testes:	35–46	About 130	137–171	25–35
Cirrus sac:	Elongate, slightly lobed	Pyriform	Retort-shaped	Elongate, lobed
Ovary:	Obliquely ovoid, slightly lobed	Rounded or transversely oval	Transversely elongate	Rounded, transversely ovoid, or slightly lobed

mainly in being relatively wider, in the different distribution of the cuticular spines, in the ratio of the length of the oesophagus to the length of the body, in the number of testes, and in the shape of the cirrus sac and ovary.

SPECIFIC CHARACTERS OF *A. MARGOLISI* N.SP.

Body greyish white, elongate, 3.82–4.96 mm in length, 1.14–1.39 mm in breadth, with lanceolate extremities; spines, 0.017 mm in length, arranged in groups of 20–40, groups about 0.022 mm apart; spines absent from the greater part of the dorsal body surface; oesophagus 1.07–1.43 mm in length; anterior limbs of H-shaped intestine approximately one-third the length of the posterior limbs; dorsal genital pore situated midway between midline and left lateral body margin; 35–46 testes; cirrus sac elongate and slightly lobed; ovary, obliquely ovoid and slightly lobed; metraterm runs obliquely backwards parallel to cirrus sac.

The holotype of *A. margolisi* n.sp. is deposited at the British Museum (Natural History), London, U.K. The paratypes are in the author's collection.

COMMENTS ON *APOROCOTYLE* FROM OTHER HOSTS

Zhukov (1960) found specimens of an aporocotylid in the hearts of 9 of 19 *Theragra chalcogramma* caught off the Putyatin Islands (Kurile Islands) in the Pacific Ocean. He identified these as *A. simplex*. Furthermore, he makes *A. orientalis* Yamaguti, 1934, a synonym of *A. simplex* without giving full reasons; the cuticular spination in the two species is not compared. In the writer's opinion the nature and distribution of the cuticular spines may prove to be of specific value in the genus *Aporocotyle*. It is interesting that Zhukov's figure of "*A. simplex*" shows the oviduct entering directly into the central chamber. In a specimen of an aporocotylid from the gills of a *T. chalcogramma* caught in the Burke Channel, British Columbia, the oviduct bears the same relation to the yolk duct and central chamber as it does in *A. spinosicanalis* and *A. margolisi* n.sp. An illustration of the main features of this fluke is given (Fig. 9). It is hoped that further specimens will become available as the one on which this drawing is based is now damaged. It has 93 testes, whereas Zhukov's "*A. simplex*" shows 90–100, and Yamaguti's *A. orientalis* shows 138 in the type and 137 and 171 in the two co-types. Odhner found about 130 testes in *A. simplex* (but figures only about 106 in his original description (Odhner, 1900) and only about 113 in a later paper (Odhner, 1911)). McFarlane (1936) found an aporocotylid that he identifies as *A. simplex* in the blood of a rock cod (*Sebastodes* sp.) caught in Departure Bay, British Columbia. He notes that the fluke did not occur commonly, but that Guberlet, in a personal communication, informs him that "this parasite is commonly found in the same genus of fishes in Puget Sound." The figure of this aporocotylid shows 45 testes.

Strelkov (1960) apparently follows Zhukov (1960) in identifying as *A. simplex* specimens from *Theragra chalcogramma* caught in Avachin Bay and in the East Kamchatsk region (Pacific Ocean). Isaichikov (1928, 1933) also assigns to *A. simplex* flukes collected from *Hippoglossoides platessoides* caught in the Barents Sea.

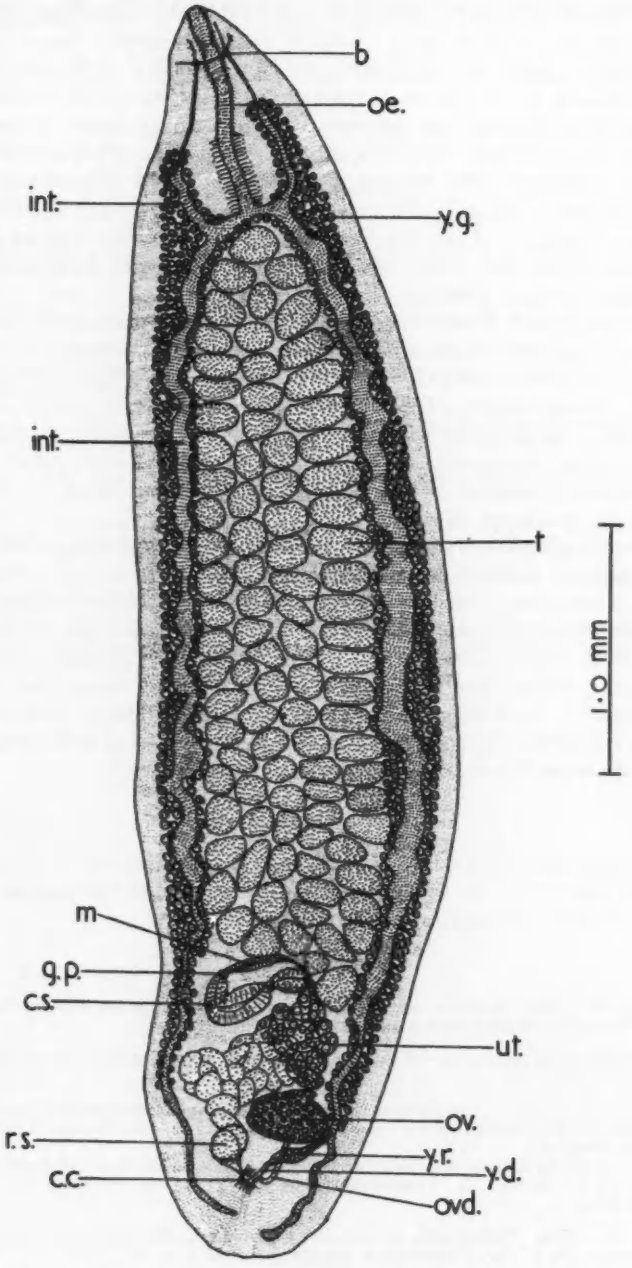

FIG. 9. *Aporocotyle* sp. from *Theragra chalcogramma*. (For key to abbreviations see caption for Fig. 3.)

An interesting record is that of Ronald (1960). He found one specimen of an aporocotylid, identified as *A. simplex*, in the intestinal mesentery of an *H. platessoides* caught on the Miscou Bank in the Gulf of St. Lawrence. No eggs were present in the uterus. Ronald states that "the cuticle carried spines, the largest of which were on the ventral surface, in groups of 11 to 17. The number of testes present in this specimen was 143 (a greater number than previously recorded)." The habitat recorded by Ronald is interesting in that Short (1954) notes a questionable record of Bazikalova (1932) who lists "Aporocotylidae (?) gen.sp." from the intestine of *Raja radiata*. The blood flukes *Deontacyclix ovalis* and *Plehniella coelomicola* apparently live in the host's body cavity (Manter, 1940; Szidat, 1951).

Both Isaichikov (1928, 1933) and Ronald (1960) claim *H. platessoides* to be a new host record for what they identify as *A. simplex*; both authors appear to have overlooked Odhner's (1911) legend to a figure of *A. simplex* in which *Drepanopsetta* (=*Hippoglossoides*) *platessoides* is named as a host.

Although the foregoing suggests that the number of testes may have little taxonomic value, it is perhaps significant that *A. spinosicanalis* and *A. margolisi* n.sp., both from species of *Merluccius*, each have less than 50 testes; the former shows 25–35, the latter 35–46.

The various aporocotylid flukes discussed above and assigned by authors to *A. simplex* are from fish representing very different families (*Cottunculus* — Cottidae; *Sebastodes* — Scorpaenidae; *Theragra* — Gadidae; *Hippoglossoides* — Hippoglossidae) and caught in different zoogeographical areas (*Cottunculus* — Asiatic North Pacific; *Sebastodes* — American North Pacific; *Theragra* — American and Asiatic North Pacific; *Hippoglossoides* — Boreal and American North Atlantic). It is suggested therefore that these blood flukes should be left undetermined as "*Aporocotyle* sp." until further material becomes available, and that *A. orientalis* should be retained as a valid species.

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Cardiovascular Dynamics in Swimming Adult Sockeye Salmon[1,2,3]

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ABSTRACT

Dorsal aortic blood pressure, heart rate, and oral water pressure were recorded in adult sockeye salmon (*Oncorhynchus nerka*) while they were resting in darkened aquaria or swimming in an illuminated tunnel-respirometer. Pressures were recorded on an oscillograph attached to pressure transducers connected with cannulae in the dorsal aorta and oral cavity. The velocity of the water in the swimming chamber was increased in stepwise fashion every 30 min until the fish stopped swimming and were swept against an electrified grid. At the cessation of swimming, which was considered as the onset of fatigue, water velocity was decreased to a minimum which permitted the fish to rest and dorsal aortic blood pressure, heart rate, and oral water pressure were recorded for about 1 hr as they returned toward resting levels. Resting values were 44/38 mm Hg (systolic/diastolic) and 49 heartbeats/min. At maximal swimming speed dorsal aortic blood pressure was 55/49 mm Hg and heart rate was 83 beats/min. No abrupt changes in cardiovascular function occurred at the onset of fatigue.

A number of morphological (length and weights of the body and ventricle) and cardiovascular (pressures, rates) variables were measured in six adult sockeye salmon. A significant correlation was found between hematocrit, ventricle weight, and heart rate; a change in one apparently caused adjustments in the other two in order to maintain a constant supply of oxygen to the tissues during subfatigue levels of activity.

The effects of removal and replacement of blood or isotanic saline in the dorsal aorta were observed in three adult sockeye salmon. Removal of blood produced a proportionate decrease in blood pressure and an increase in heart rate. Neither dilution of the blood by replacement with saline nor increase in blood volume from addition of saline had any significant effect on heart rate and blood pressure.

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INTRODUCTION

THE MARINE LIFE of the Pacific salmons is characterized by long migrations, which culminate in freshwater spawning. Salmon travel about 1–1½ miles per hour at sea. In the journey upstream, they increase their rate of swimming considerably to ascend the swifter currents of some streams. There have been many investigations of the biochemical and endocrinological changes and the energy requirements of salmon during migration (Idler and Clemens, 1959) but none on cardiovascular dynamics.

Dorsal aortic blood pressure and heart rate have been measured in restrained or anesthetized chinook salmon (*Oncorhynchus tshawytscha*) by several investigators. Greene (1904) recorded a range of 38–58 mm Hg and a mean of 46 mm Hg for blood pressure in restrained fish and a range of 28–72 heartbeats/min with an average of 49 beats/min. A kymograph with a mercury monometer was used to record blood pressure. Using oscillographs and pressure transducers, Robertson et al. (1966) measured blood pressure in the ventral and dorsal aortas of anesthetized chinook salmon obtained from the same geographical area as those observed by Greene (1904). An average dorsal aortic blood pressure of 44/37 mm Hg was observed; however, the flow of water over the gills was stopped when recording blood pressure which may have affected the level. From the low heart rate (average 18 beats/min), bradycardia was apparently induced.

Brett (1964) studied the respiratory metabolism of salmon at several swimming velocities. The fish were induced to swim against a stream of water pumped through a plastic tube having a bore of 4.5 inches (11.25 cm). Since small adult sockeye salmon (*Oncorhynchus nerka*) performed well in this device, it was proposed to study the cardiovascular dynamics if a recording system could be attached to the fish that would withstand their strenuous activity. Smith and Bell (1964) developed a technique for cannulation of the dorsal aorta of salmonids which filled this need. In late July, August, and September of 1964, 1965, and 1966, cardiovascular observations were conducted on (1) sockeye salmon as they rested or swam at different velocities and (2) sockeye salmon in which hemodynamic changes were induced by removal and replacement of blood. These observations are reported in this paper.

MATERIALS AND METHODS

OBSERVATIONS OF RESTING, SWIMMING, AND FATIGUED SALMON

The experimental fish were adult sockeye salmon which had been trapped from the headwaters of the Stamp River near Great Central Lake, Vancouver Island, British Columbia. The fish were transported by truck in an oxygenated, iced tank from the place of capture to the Biological Station at Nanaimo, where they were held in running freshwater at 13–16 C in large circular tanks without being fed. Sea water (salinity, 25‰) was added to the tanks twice a week to prevent the growth of fungus on the fish.

Each fish was prepared for observations as follows. The fish was taken by net from the holding tank and anesthetized with M.S.-222. When it became inactive it was transferred to an operating table and supported ventral side up. A chilled, oxygenated solution of M.S.-222 and freshwater (100 mg/liter) was circulated over the gills. Using the method of Smith and Bell (1964), a cannula was inserted through the nasal cartilages into the mouth, thence it was led posteriorly and implanted in the dorsal aorta. A second cannula was inserted through the nasal region with its opening in the mouth cavity. The fish, still anesthetized, was then placed in the respirometer in a gentle (0.3–0.6 ft/sec) stream of water to facilitate respiration during recovery. The fish's two cannulae were led out of the respirometer through a standpipe and were each connected to a Sanborn or Statham pressure transducer, which in turn was connected to either a Sanborn or a Beckman oscillograph. Polyethylene tubing with a bore of 0.030 inches (PE-60) and a length of about 5 ft (1.5 m) was used for the cannulae. It provided negligible attenuation below about 30 pressure pulses/sec. The cannulae were filled either with Cortland saline (Wolf, 1963) or with a solution of heparin and water in the proportion (1000 IU/ml). Figure 1 shows the equipment in operation.

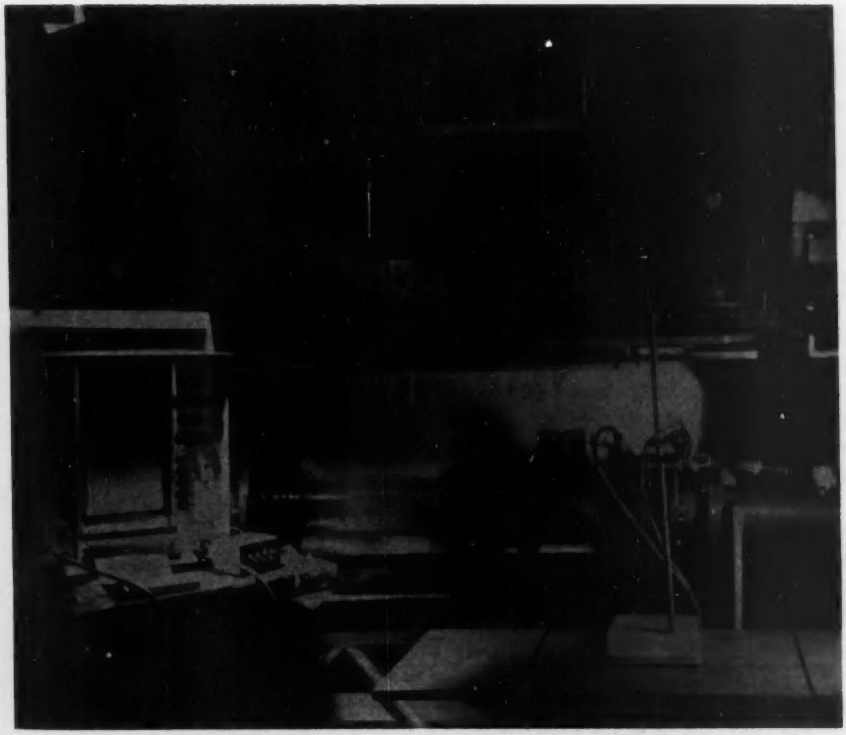

FIG. 1. The swimming chamber of the respirometer during recording of blood pressure in a swimming sockeye salmon.

Ventilating movements usually began immediately after the fish was placed in the respirometer. The fish righted itself within a few minutes and learned to stay upstream from an electrified grid (approximately 5 v a-c) during a 30-min introductory period. In the relatively small tunnel diameter the larger fish had difficulty in remaining upright at low water velocities, so the velocity was usually increased during the second hour of recovery from anesthesia to about 0.8 ft/sec (24 cm/sec). Because of these problems with equilibrium, minimum values for heartbeat and blood pressure were obtained from fish resting quietly in darkened aquaria or fish which had remained in the respirometer overnight without difficulty. Fish observed in the aquaria were never placed in the respirometer, consequently there are no data on heartbeat and blood pressure of these fish while actively swimming. Once the fish was actively swimming in the respirometer, it appeared to have no difficulty in maintaining equilibrium. Excellent performance was obtained in a new respirometer (1966) having a bore of 8.0 inches (20.3 cm). All observations in both respirometers were conducted in freshwater at 15 C.

Approximately 2 hr after the fish was put in the respirometer, water velocity was increased just sufficiently to cause the fish to swim. Velocity was then increased by small sequential increments, usually 0.2 ft/sec (6.0 cm/sec) metered velocity, until the fish could no longer maintain position upstream from the electrified grid. This event was considered as the onset of fatigue. At this point, water velocity was reduced to 0.3 ft/sec (9.0 cm/sec) and measurements of oral water pressure and dorsal aortic pressure continued. Observations were usually conducted for no more than an hour after the onset of fatigue because the fish usually recovered enough to become active after that time, although complete recovery in fingerling sockeye has been shown to take about 3–4 hr (Brett, 1964).

Preliminary experiments indicated that heart rate and rate of ventilation changed markedly during extreme reduction in environmental oxygen levels. These changes were similar to those reported by Holeton and Randall (MS, 1967) in rainbow trout. Heart rate and rate of breathing also decreased when oxygen level was considerably higher than air-saturation level. Since the experiments were not planned to include investigation of such changes, care was taken to maintain the oxygen level in the respirometer within 80 to 120% of the air-saturation level at 15 C, the range in which little change in hemodynamics was observed from changes in oxygen saturation.

Since the adult fish occupied a relatively large portion of the cross-sectional area of the tunnel and consequently increased the velocity of the water surrounding the body, no means of determining the true velocity of the water for each fish was afforded. Since swimming speed is directly related to the frequency of tail beat (TBF) (Bainbridge, 1958), TBF was counted visually as a measure of the average swimming effort.

Data were analyzed in several ways. Dorsal aortic blood pressure and oral water pressure were recorded by oscillograph for 1–3 min during the first, middle, and last parts of each 30-min period of swimming at a given velocity.

TBF was counted at least once during each 30-min period and often with each recording. From the oscillograms, one minute of record was chosen which showed no unusual pressures (which sometimes occurred during spontaneous vigorous activity). The pressure pulses indicating the heart rate were counted and typical blood pressure was noted (Fig. 2). These records show to some extent the variability in blood pressure in the dorsal aorta which occurred during excessive activity and which made selection of appropriate sections necessary. Observations of the immature male "jack" sockeye salmon (700–800 g) were carried out in exactly the same way as those of the adults (1200–1800 g).

Heart rate and blood pressure were generally higher early in the 30-min period than later, as shown in Fig. 3. In such cases and when there was no change between observations, data were taken for analysis from the last observation during the half-hour. In cases when there was variability and no indication of any stabilizing tendency toward the end of the period, all three observations were averaged. Thus, the data were screened to present a conservative record of cardiovascular changes.

Data on oral water pressures were treated in the same manner. These data are not reported except among the correlations because they were of value mostly for determining whether an inactive fish was in a resting state or not. Oral pressure pulses had greater amplitude and frequency in inactive, fatigued fish than in rested fish. When swimming at higher velocities, most fish held their mouths open continuously and no oral pressure *pulses* were produced (Fig. 2).

Other morphological and physiological variables were measured in 6 of the 19 fish to determine their significance in cardiovascular dynamics (Fig. 6, 7). Length was measured from the tip of the head to the fork of the tail to the nearest 5 mm. Weight was measured to the nearest gram while the fish was anesthetized. Hematocrit was determined from blood which had been centrifuged in heparinized capillary tubes in a hematocrit centrifuge. Pulse pressure was measured as the difference between the maximum and minimum pressures recorded from the mouth with each opening and closing. Ventilation rate was measured as the number of closures of the mouth per minute.

Experimental Changes in Volume and Concentration of Blood Constituents

Experiments were performed on three adult sockeye salmon to ascertain the effects of changes in the volume and viscosity of the blood and concentration of erythrocytes, and to determine whether any of these factors might limit normal swimming performance. Each fish, after cannulation of the dorsal aorta and the oral cavity, was placed in the respirometer having an 8-inch (20-cm) diam swimming chamber. The water velocity was kept at 2 ft/sec (64 cm/sec) to induce the fish to swim slowly (TBF was 106–108/min), but some fish rested against the downstream grid (which was not electrified). From 10 to 20 ml of blood was removed periodically through the cannula, after which

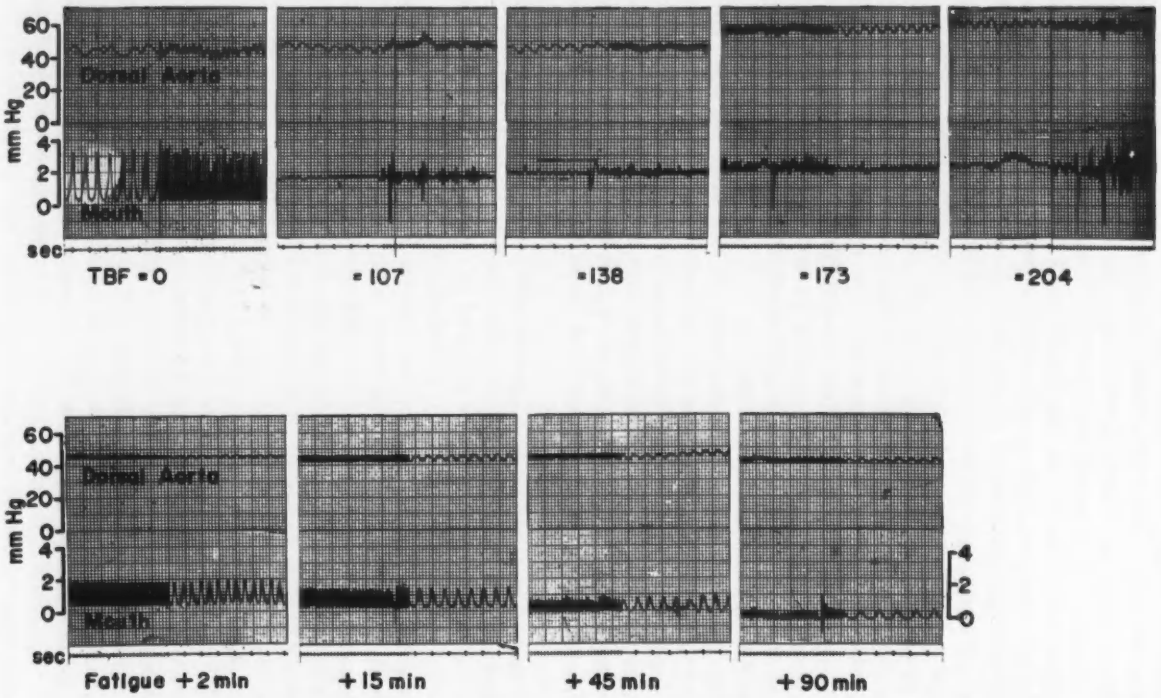

FIG. 2. Samples of typical oscillograph records of dorsal aortic blood pressure and oral water pressure in an adult sockeye salmon swimming in the tunnel respirometer. Recording speed was increased from 1 mm/sec to 5 mm/sec in a portion of each record to clarify the shape of the wave forms.

dorsal aortic blood pressure and heart rate were recorded for 10–30 min. Depending on the type of experiment, blood was replaced with blood previously removed from the same fish and stored in clean glass containers packed in ice, or it was replaced with Cortland saline (Wolf, 1963). Responses to hypo- and hypervolemia and to dilution of blood were observed.

In the first experiment 15 ml of blood was removed, dorsal aortic blood pressure was recorded for 10–20 min, the blood was replaced with saline, and pressure was recorded for 10–20 min. This procedure was repeated six times.

In the second experiment 15 ml of blood was removed periodically without replacement and dorsal aortic blood pressure was recorded after each removal.

In the third experiment, a saline injection increased the blood volume to greater than normal and the dorsal aortic pressure was recorded. After this, additional blood samples were removed and replaced with saline.

RESULTS

Observations of Resting, Swimming, and Fatigued Salmon

Immature "jack" sockeye salmon

The observations of dorsal aortic pressure and heart rate in "jack" salmon illustrate certain general features of the hemodynamics of salmon. Each of the three observations during the half-hour period of swimming at a given velocity of a "jack" sockeye is shown in Fig. 3. Higher values tended to occur

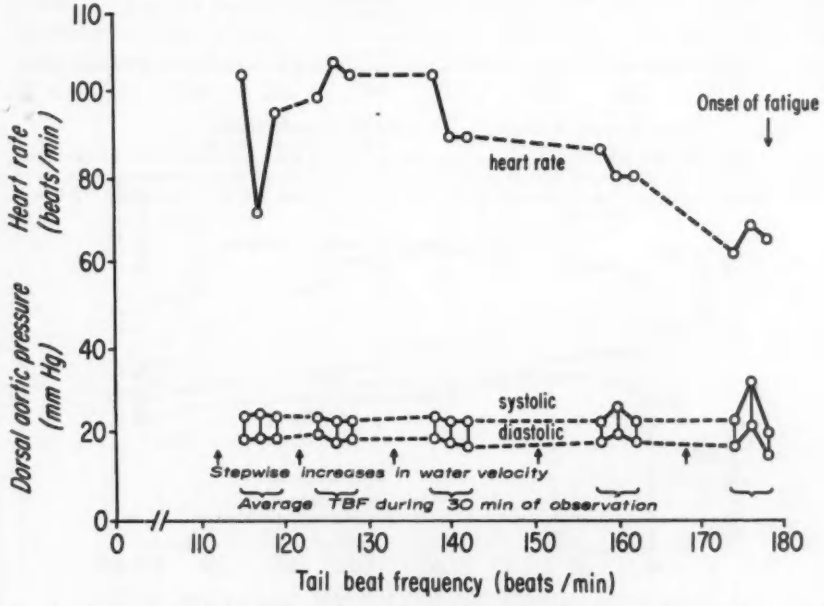

FIG. 3. Average heart rate and dorsal aortic blood pressure in an immature male ("jack") sockeye salmon during the early, middle and late portions of 30-min periods of swimming until the onset of fatigue. Water velocity was increased by a small increment and maintained at the higher level for the next 30 min. (Size of fish about 750 g, temperature, 15 C.)

early in each 30-min period. Average blood pressure remained constant until maximal swimming velocity was reached. Heart rate decreased with increasing frequency of tail beat only in "jack" sockeye. Unfortunately, the cannula came out of the dorsal aorta in this fish shortly after the onset of fatigue, so no observations of recovery after fatigue were possible.

Figures 4 and 5 show observations (averaged as described in the section Materials and Methods) of a "jack" sockeye salmon during swimming and after

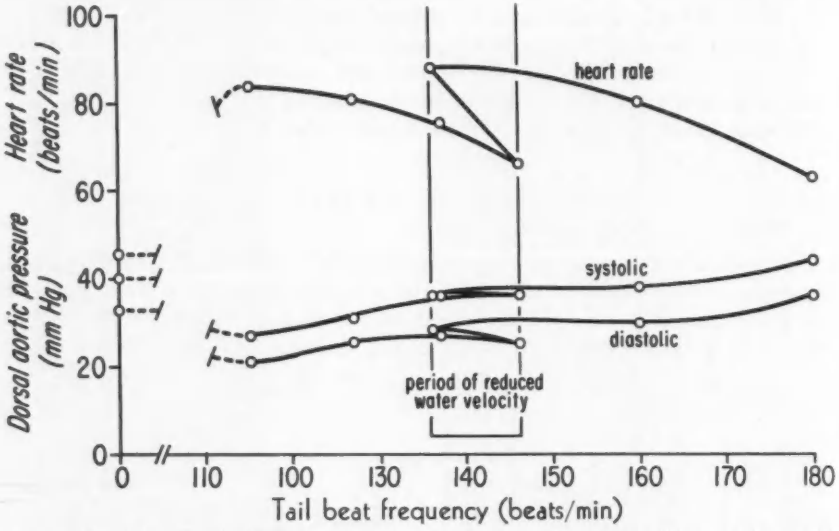

FIG. 4. Records of a "jack" sockeye salmon tested under the same conditions as the "jack" sockeye salmon whose records appear in Fig. 3, except that water velocity was reduced for one of the 30-min periods rather than increased. (Size of fish about 750 g, temperature, 15 C.)

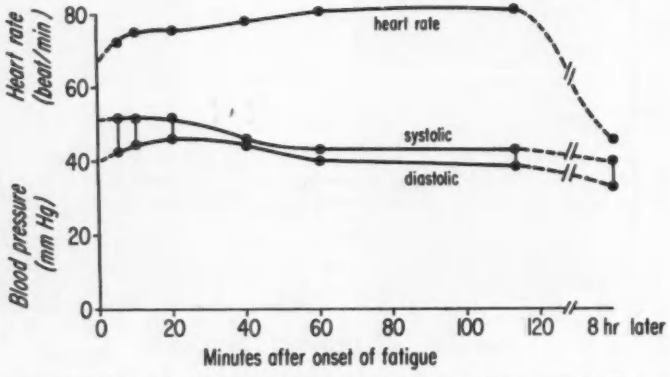

FIG. 5. Records from the "jack" salmon whose records appear in Fig. 4 after the onset of fatigue.

the onset of fatigue. The changes in heart rate during the swimming period were much as before, but dorsal aortic blood pressure steadily rose. Heart rate returned almost to the same level it reached at the previous swimming velocity during a half-hour of swimming at only slightly reduced velocity. After the onset of fatigue and reduction of water velocity so that the fish could maintain position in the tunnel without swimming, heart rate and blood pressure slowly returned to values before swimming began (Fig. 5). This fish remained in the respirometer overnight and the last observation was made the following morning.

MATURE SOCKEYE SALMON

Data from 10 mature sockeye salmon of both sexes are shown in Fig. 6 and 7. Heart rate increased with the lower and intermediate increments of water velocity, but tended to remain constant at high velocities. On the other hand, blood pressure stayed nearly constant until after heart rate stabilized. The onset of fatigue usually followed a small, gradual rise in dorsal aortic blood pressure.

There was a wide range of standard deviations in heart rate among these fish during successive periods of swimming at increasing velocities. During the first 30-min period of swimming, heart rate was erratic (as in Fig. 3), but it became less so during the second period. Thus, standard deviations were high at first and became less as the fish became more accustomed to swimming in the respirometer. During periods of swimming at still higher velocities, some fish fatigued more rapidly and reached maximal heart rate earlier than others. Thus

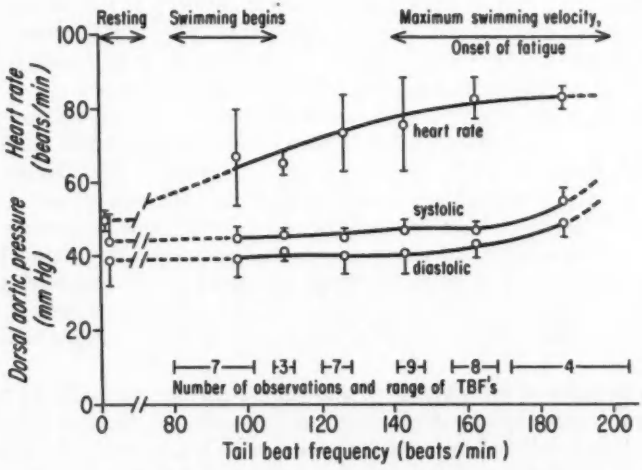

FIG. 6. Average heart rate and dorsal aortic blood pressure (with one standard deviation shown) in 10 adult sockeye salmon during stepwise increases in swimming velocity. Average size and other measurements of these fish are shown in Table I. (Temperatures, 15 C.)

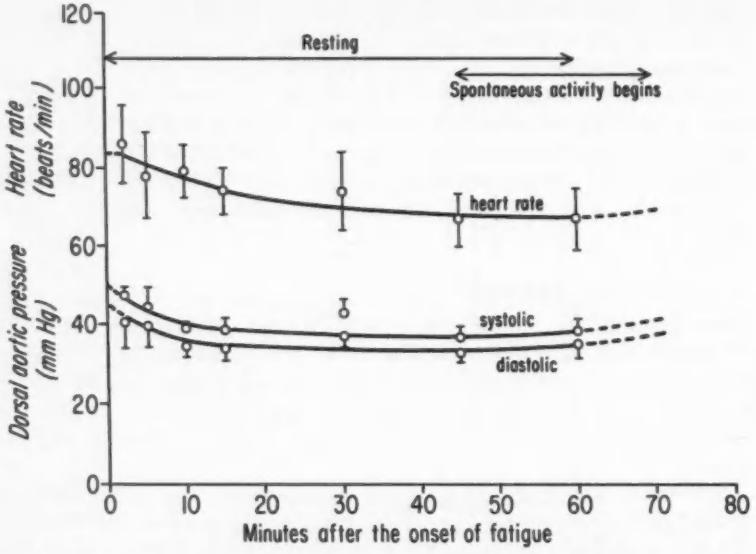

FIG. 7. Average heart rate and dorsal aortic blood pressure (with one standard deviation shown) in 10 sockeye salmon (with records in Fig. 6) after the onset of fatigue. Oscillations in both heart rate and blood pressure were smoothed because they were not conspicuous in individual fish. (Temperature, 15 C.)

the standard deviation increased again. Finally, at very high velocities each fish seemed to reach a consistent maximal heart rate. The considerable variability in resting dorsal aortic blood pressure among these fish probably reflects the difficulty of inducing normal fish to stop swimming completely.

After the onset of fatigue and reduction of water velocity in the swimming chamber, heart rate and dorsal aortic blood pressure declined to resting values (Fig. 7). If instead of a smoothed line a line is drawn to follow the mean points exactly, some interesting oscillations appear in both heart rate and dorsal aortic blood pressure. Such oscillations could result from a variety of cyclic hemodynamic changes which may occur during recovery from fatigue. However, the mean oscillations are relatively small compared to the standard deviations and therefore not statistically significant. Thus a smoothed line is the best approximation of the relation until there is established evidence for an oscillating line.

Various pairs of morphological and cardiovascular variables in 6 sockeye salmon were examined for correlation (Table I) with the intention of identifying easily measured combinations which could serve as an index to other cardiovascular characteristics of salmon. Some of the correlations simply confirmed relations already apparent from casual observations: heart rate and ventilation rate increased with TBF; systolic pressure was highly correlated with diastolic

TABLE I. Correlations among several morphological, cardiovascular, and ventilation factors in adult sockeye salmon.[a]

	Mean ± 1 SD	Weight (*g*)	Length (*cm*)	Hemato-crit (*% RBC*)	Weight of ventricle (*% of body weight*)	Frequency of tail beat (*beats/min*)	Heart rate (*beats/min*)	Dorsal aortic pressure, systolic (*mm Hg*)	Dorsal aortic pressure, diastolic (*mm Hg*)	Pulse pressure (*mm Hg*)	Ventilation, rate (*closures/min*)
1. Weight	1652 ± 129	–	–	–	–	–	–	–	–	–	–
2. Length	55.9 ± 19.0	.83[c]	–	–	–	–	–	–	–	–	–
3. Hematocrit	30.4 ± 1.0	−.22	−.37	–	–	–	–	–	–	–	–
4. Weight of ventricle (% body weight)	.134 ± .005	.34	.12	−.12	–	–	–	–	–	–	–
5. Frequency of tail beat	90.4 ± 14.6	−.84[c]	−.68[c]	.32	−.41[b]	–	–	–	–	–	–
6. Heart rate	65.0 ± 3.9	**−.51[c]**	**−.47[c]**	**.46[c]**	**−.54[c]**	**.76[c]**	–	–	–	–	–
7. Dorsal aortic pressure, systolic	45.5 ± 1.1	.03	.12	−.10	.12	.21	.15	–	–	–	–
8. Dorsal aortic pressure, diastolic	40.0 ± 1.0	.35[b]	.07	.28	−.11	.29	.40[b]	.90[c]	–	–	–
9. Pulse pressure	5.4 ± 0.7	.01	−.14	.41[b]	**.52[c]**	−.18	**−.56[c]**	.20	−.24	–	–
10. Ventilation, rate	79.4 ± 4.1	−.49	−.32	.23	**−.55[b]**	**.60[b]**	**.65[c]**	−.34	−.08	−.43	–
11. Ventilation, pressure (pulse height)	1.6 ± 0.2	.34	.27	.40	.38	−.37	−.06	.23	.28	−.05	.01

[a]Correlations of particular physiological importance are in boldface type (*see discussion in text*). For items 1–9, $n = 31$, p .05 = .35, p .01 = .45. For items 10–11, $n = 16$, p .05 = .50, p .01 = .62. b = significant at $p = .05$; c = significant at $p = .01$.

pressure in the dorsal aorta; larger fish had a lower TBF. Other correlations accorded with physiological principles established for the higher vertebrates: pulse pressure decreased as heart rate increased; heart and ventilation rates decreased as body or ventricle weight increased; pulse pressure increased with ventrical size but absolute pressure did not.

The lack of significant correlation between some of the parameters recorded was also physiologically significant. There was little correlation between blood pressure and any of the factors which might correlate with swimming activity — TBF, heart rate, or ventilation rate. Hematocrit, sometimes thought of as a useful indicator of a fish's general well-being, was not correlated significantly with any of these factors except negatively with heart rate. The weight of the ventricle was only slightly correlated with the weight or length of the body; it appears that the size of the ventricle is a function of some factor besides general rate of growth.

Experimental Changes in the Volume and Concentration of Blood Constituents

In the fish from which blood was removed periodically and an equal volume replaced with saline (Table IIA), the hematocrit fell from 34% (the initial value) to 1% during a 2-hr period. After each removal of blood, heart rate and ventilation rate rose while dorsal aortic blood pressure fell. All three returned to nearly normal levels after the blood was replaced with saline. However, after the hematocrit had fallen to 16%, the heart rate rose from the low 60's to the mid 80's and remained there with little response to further changes in volume of the blood or dilution. Dorsal aortic blood pressure decreased slowly until the hematocrit fell to 10% and then decreased at a greater rate as hematocrit decreased further. Even when the hematocrit was only $\frac{1}{10}$th of the original, blood pressure was still two-thirds of the value at the start. In spite of the dilution of plasma proteins and red blood cells, the shape of the pressure pulse recorded in the dorsal aorta appeared normal except for greater span between systolic and diastolic pressure.

When blood was removed without replacement (Table IIB), blood pressure decreased in proportion to quantity of blood removed. Heart rate increased at first, but decreased below normal levels after removal of additional blood. The slow rate of return toward normal heart rate, dorsal aortic blood pressure, and ventilation rate suggests that complete recovery would require many hours, perhaps days. (Observations were not continued for that period of time.)

Dorsal aortic blood pressure and heart rate returned to near normal levels when most of the blood removed was replaced with saline. The large pulse pressure probably reflects the reduced resistance to flow of the diluted blood.

In the third adult sockeye salmon tested, only two of several volume changes (data not shown) produced effects not seen in the first two fish. First, the fish stopped swimming (TBF was 106 to 108) upon removal of as little as 10 ml of blood and rested its tail against the downstream grid but it began

TABLE II. Effects of hematological changes on hemodynamics in adult sockeye salmon.

TABLE II. Effects of hematological changes on hemodynamics in adult sockeye salmon.

Volume change (± *ml, saline or blood*)	Blood volume[a] (*ml, total*)	Hematocrit[b] (*% RBC*)	Heart rate (*beats/min*)	Dorsal aortic blood pressure (*systolic/diastolic, mm Hg*)	Ventilation rate (*oral pressure pulses/min*)	Interval between volume change and recording (*min*)
A. Effects of blood dilution (adult male, 57 cm, 1800 g, 15 C)						
None	90	34	58	68/64	52	
−15, blood	75	34	86	42/40	60	5
+15, saline	90	26	63	64/60	57	5
−15, blood	75	26	84	34/22	60	5
+15, saline	90	16	60	64/58	Not recorded	5
−15, blood	75	16	87	34/32	66	5
+15, saline	90	10	84	59/55	56	5
−15, blood	75	10	87	28/26	72	5
+15, saline	90	6	84	56/52	48	5
−15, blood	75	6	87	26/24	75	5
+15	90	4	84	42/32	75	5
−15, blood	75	4	86	27/24	92	5
+15, saline	90	1	81	44/37	60	5
B. Effects of blood loss (adult female, 58 cm, 1930 g, 15 C)						
None	96	31	69	68/62	66	
−15, blood	81	30	90	42/38	81	1
None	81	30	86	48/46	72	7
−10, blood	71	30	90	28/26 to 42/38[c]	78	3
None	71	30	82	44/40	64	16
−15, blood	56	22	54	26/24	69	1
None	56	16	72	31/28	72	15
−15, blood	41	11	50	14/12	Too faint	1
None	41	10	70	20/18	62	12
− 5, blood	36	10	approx 46 (faint)	15/14	58 (faint)	1
+40, saline	76	?	66	58/50	70	2
None	76	3	78	52/45	86	30
C. Effects of increased blood volume (adult female, 54 cm, 1680 g, 15 C)						
None	84	25	56	56/48	94	
+20, saline	104	17	54	56/48	111	10

[a]Initially estimated as 5% of body weight; succeeding changes are cumulative, based on amount of volume change.
[b]After the saline or blood injected had circulated for approximately 10 min.
[c]Large, slow oscillations in dorsal aortic pressure occurred.

swimming when the blood was replaced with either blood or saline. The withdrawal and replacement were repeated three times with the same results each time.

The second notable observation was the lack of effect from a moderate increase in blood volume (Table IIC). Neither heart rate nor dorsal aortic blood pressure changed appreciably. The decreased hematocrit probably accounted for the increase in breathing rate. No attempt was made to find the point at which increased blood volume would begin to influence heart rate or blood pressure.

DISCUSSION

The range of dorsal aortic blood pressure recorded by Robertson et al. (1966) in chinook salmon corresponds with that observed by us in sockeye salmon. The wide range of values reported by Greene (1904) for heart rate and dorsal aortic blood pressure in restrained, unanesthetized chinook salmon support our contention that increase in heart rate is largely the means by which salmon increase heart output during activity. Some fish undoubtedly struggled against restraint and their heart rates and blood pressures are comparable to those of fish swimming until fatigued in our respirometer. The responses recorded by Stevens and Randall (MS, 1967) and Randall et al., (1965) in rainbow trout (*Salmo gairdneri*) differ little from those observed by us in sockeye salmon; resting heart rate is slightly higher and dorsal aortic blood pressure slightly lower.

On the other hand, Johansen (1962), who recorded blood pressure and flow in the ventricle and bulbus of Atlantic cod (*Gadus morhua*), reported that the primary response to activity was a rapid increase in cardiac output because of increased blood pressure without any increase in heart rate. He attributed the rapid increase in stroke volume to greatly increased venous return. Conversely, Randall (1966) observed that cardioacceleration was the chief means of increasing cardiac output in the tench (*Tinca tinca*). If the responses of sockeye salmon to blood withdrawal (decreased blood pressure and increased heart rate) are interpreted as decreased output followed by homeostatic response then salmon are as sensitive to changes in venous return as cod. The response of salmon to changes in venous return, however, primarily affects heart rate rather than blood pressure. The explanation might lie in a difference in vasomotor ability between cod and salmon. Perhaps salmon have shunts in the gills which allow rapid increase in cardiac output without increased resistance, and cod do not. Whether these are two separate responses or different degrees of the same response remains to be seen.

In the two immature male "jack" sockeye salmon observed, heart rate slowly declined with increased swimming velocity. In one of these fish, dorsal aortic blood pressure nearly doubled. There was no indication that the two immature fish were in an abnormal condition, and we cannot explain why they differed in this respect from adult sockeye salmon.

No abrupt changes occurred at the onset of fatigue which might suggest that cardiovascular failure caused the sudden cessation of swimming. Cardiovascular changes, if any, at the onset of fatigue did not correspond in degree with the suddenness of the onset of fatigue. On the other hand, since few fish could be stimulated to swim after the onset of fatigue, it marked some kind of major physiological event. Generally, the onset of fatigue was not predictable either from the cardiovascular records or from the general behavior of the fish.

From the extensive work of Black (1957), it appears unlikely that the accumulation of lactate during the swimming period caused the onset of fatigue since blood lactate did not reach maximum until about 30 min after its occurrence. It is our belief that the fish ceased swimming before serious cardiovascular malfunction occurred, or that the fish collapsed because it became unconscious. Unconsciousness is suggested as a possible occurrence because no response was obtained from fatigued fish except a few muscle twitches when the voltage in the grid was increased. On the other hand, most fish moved upstream from the grid, sometimes without any electrical stimulation, when the velocity of the water was reduced to 0.3 ft/sec so that the fish could rest on the bottom of the tunnel without being displaced by the water current. Therefore, unconsciousness did not last very long if it did occur.

The correlations among the various morphological and cardiovascular measurements (Table I) did not provide much assistance in identifying factors limiting swimming velocity, but they suggested the source of some of the great variability in blood volume (Smith, 1966), hematocrit, and ventricle weight (Table I). It appears from the correlations that oxygen consumption is relatively constant under given conditions of temperature and activity and that cardiac output adjusts to meet this oxygen demand. Given blood of a particular oxygen-carrying capacity, either heart rate and blood pressure increase to meet short-term demands for increased cardiac output, or ventricle size and, presumably, stroke volume eventually increase over long periods of elevated cardiac output, whereas heart rate and blood pressure readjust to normal levels.

Records of blood pressure (L. Smith, unpublished results) from sockeye salmon subjected to such low levels of environmental oxygen that they lay on their backs in the respirometer (presumably they were unconscious) show that the heart continues to function long after loss of equilibrium and, presumably, loss of consciousness. Therefore, our belief that general hypoxia of the tissues, perhaps cerebral hypoxia especially, was the major cause of the sudden loss of swimming ability seems warranted. Brett (1964) showed that fish can expend temporarily more energy in "burst of activity" than is recorded during their simultaneous oxygen consumption. Thus the oxygen-carrying capacity of the circulatory system is exceeded and hypoxia of the tissues in some degree must follow. The brain seems most likely to show hypoxic effects first, rather than the cardiovascular or muscular systems.

The experiments on blood removal and dilution suggest several important characteristics of salmon circulatory systems. First, maintenance of normal

blood volume, and probably venous return, is an important factor in controlling cardiac output. On the other hand, moderate increase in blood volume has little effect on cardiac output, as in man (Robinson et al., 1966). Second, salmon compensate rather slowly for loss of blood. They would be at a considerable disadvantage in eluding a predator for many hours after injury or other trauma affecting blood volume, such as the shock after capture and tagging (Smith, 1965). The gradual increase in blood pressure (presumably, increase in blood volume as well) and drastic decrease in hematocrit observed between the last withdrawal of the blood and the first injection of saline indicate that cell-poor or cell-free fluid was entering the central circulation. This fluid could have been water from the environment which entered the blood across the gill epithelium or extracellular fluid which moved centripetally by peripheral vasoconstruction, or both. Lastly, the concept of the blood of fish as an unchanging, uniformly distributed, single pool, which most of the workers who measured blood volume in fish apparently assumed (Smith, 1966), needs thorough investigation.

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The Pituitary Gland of the Sockeye (*Oncorhynchus nerka*) During Sexual Maturation and Spawning[1]

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ABSTRACT

This communication presents the results of a study of the pituitaries of 150 sockeye salmon in various stages of sexual maturation from the time they entered freshwater till after completion of spawning. In the homologue of the adenohypophysis, nine different cell types were distinguished. On the basis of changes in tinctorial and histochemical properties and relative proportions of each of these cells, it was concluded that the sockeye pituitary contains six different hormone-producing cell types. One of these probably possesses a gonadotrophic function. Degenerative phenomena in the pituitary were found in the sexually ripe and the spent salmon, but only to a limited extent. The implications of the changes in the occurrence of the six different cell types and the degenerative alterations are discussed in relation to gonad development and the death after spawning.

INTRODUCTION

There is substantial evidence that sexual maturation and spawning of Pacific salmon is accompanied by pronounced morphological and physiological changes in various organs and tissues. Robertson and his co-workers, concentrating their investigations mainly on chinook salmon (*Oncorhynchus tshawytscha*) found these changes to be of a degenerative nature in various parts of the intestinal tract, liver, spleen, thymus, kidney, and cardiovascular system. Similarly, some endocrine glands, the thyroid and the pituitary gland were reported to undergo atrophy and degeneration, whereas others apparently became hypertrophied, e.g. the pancreatic islets and the adrenal cortex (Robertson and Wexler, 1959, 1960, 1961, 1962b; Robertson et al., 1961a, b). The hypertrophy of the adrenocortical tissue in the chinook salmon was reflected in a considerable increase in plasma concentration of 17-hydroxycorticosteroids (Hane and Robertson, 1959; Robertson et al., 1961b). The results of a comparative study of the adrenal cortex in chinook (*O. tshawytscha*) and migratory and non-migratory rainbow trout (*Salmo gairdneri*) suggested that

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hyper-adrenocorticism is an important cause of the degenerative changes mentioned above, and is therefore related to the postspawning death in these species (Robertson and Wexler, 1959; Robertson et al., 1961b). Further support for this suggestion was obtained during a study on the effect of high concentrations of hydrocortisone on immature rainbow trout (Robertson et al., 1963).

In this laboratory[2] Idler and associates initiated studies on the physiology of sockeye (*O. nerka*) during upstream migration and spawning (Idler et al., 1959; Schmidt and Idler, 1962). They reported an increase in plasma concentrations of 17-hydroxycorticosteroids in migrating and spawning sockeye collected from various locations in the Fraser River system. The adrenocortical tissue of this species, also, undergoes extensive hypertrophy during sexual maturation (van Overbeeke, 1967). However, the results of recent studies indicate that these morphological changes do not always reflect a significant increase in production of corticosteroids (Fagerlund, 1967).

As has been outlined previously (McBride et al., 1963), careful analysis of the various aspects of the physiology of Pacific salmon during this stage of their life cycle and evaluation of the factors causing their death, soon after completion of spawning, is difficult because of severe stresses to which the fish are subjected in their natural environment. Our investigations were therefore concentrated on fish which were maintained under laboratory conditions, although the results were always compared with those obtained with fish from the field. Previous results, reported by one of us (McBride et al., 1963, 1965; McBride, 1966), indicate that the changes in histological structure of the thyroid, pancreas, stomach, intestine, liver, kidney, and circulatory system in sockeye, are at least partly similar to the degenerative changes described by Robertson et al. (1961a, b) for the chinook salmon. Differences were also noted and, moreover, some of the changes could be affected by feeding.

This communication describes the results of a study of the pituitary gland in sockeye, in particular its development during sexual maturation and spawning.

MATERIALS AND METHODS

Fish

Most tissues used in this study were obtained from fish which had been captured upon entering Great Central Lake. Many of these fish were transported to the laboratory, where they were held for various lengths of time, and also utilized for other studies. The method of capture and general culture procedures have been fully described elsewhere (McBride et al., 1963; McBride, 1966). In addition, smaller samples, representing sockeye in late stages of maturation were obtained from the spawning beds in Great Central Lake. Also included in this study are the results of a study of 18 sockeye, captured at the time of spawning in Genesee River, ½ mile from Owikeno Lake near the east end of the Rivers Inlet. Detailed information on the history of the various samples is given in Table I.

[2]Vancouver Laboratory of the Fisheries Research Board of Canada, Vancouver, B.C.

TABLE I. Particulars of the sockeye used in this study.

TABLE I. Particulars of the sockeye used in this study.

Date or period fish were killed[a]	Number of specimens M	F	Origin of fish[b]	Stage of sexual ripeness	Period in captivity	Period after completion of spawning
Samples 1963–64						
June 16–July 17	2	3	Entrance G.C.L.	I		
Sept. 4–25	4	3	Tanks	I, II	2–4 weeks	
Oct. 8	8	10	Genesee River	III, IV		
Oct. 22	5	2	Spawning beds G.C.L.	III, IV		
Dec. 11–24	2	3	Tanks	IV	6 months	2–7 days
Jan. 8–16		2	Tanks	IV	7.5 months	6–7 weeks
Jan. 20	1	1	Tanks	IV	7.5 months	2–4 days
Feb. 3		1	Tanks	IV	8 months	2 weeks
Feb. 11–14	5	1	Tanks	III	8.5 months	
Apr. 3–16	1	1	Tanks	IV	10 months	1–3 months
Samples 1964–65						
July 9	3	2	Barkley Sound (seawater)	I		
Oct. 1–8	5		Tanks	II, III	3 months	
Oct. 16	1	1	Tanks	IV	3.5 months	2 days
Oct. 21	2	3	Spawning beds G.C.L.	?		
Oct. 27–Nov. 18	5	5	Tanks	IV	4–4.5 months	1–7 days
Nov. 13		1	Tanks	III	4.5 months	
Nov. 20	1		Tanks	IV	5 months	
Dec. 15–Jan. 6	2	2	Tanks	IV	6 months	3–6 weeks
Samples 1965–66						
July 16	8	12	Entrance G.C.L.	I		
Sept. 10	1	4	Robertson Creek	II, III		
Sept. 22	2	4	Tanks	III	2 months	
Oct. 21–Nov. 1	6	7	Tanks	III	3–3.5 months	
Nov. 9–13.	5	8	Tanks	IV	4 months	1–12 days

[a]Data for each individual fish are known, but for briefness' sake, compiled data are presented here.
[b]All fish, taken from the tanks, were originally captured at the entrance of Great Central Lake (for details see text).

Four stages of sexual ripening were distinguished.

I. Adult, but sexually immature sea green fish. Gonad weight of the males: <1% of total body wt; of the females: <4% of total body wt.

II. Sexually maturing fish, secondary sexual characteristics developing (coloration of skin, hooked jaws). Gonad weight of the males: 1 to 3% of total body wt; of the females: 5 to 14%.

III. Sexually mature fish. Gonad weight of the males >3%; of the females >15%.

IV. Fish during spawning or after completion of spawning.

The number of fish studied, their origin, the date of killing, and the stage of maturity, as well as the time elapsed after completion of spawning, are listed in Table I.

Histological Techniques

Fish were killed by severing the spinal cord just caudal to the head. The pituitary of the sockeye is located in a depression of the base of the skull. In sexually mature fish, however, this organ is often pinched in the bone. To avoid damaging the delicate tissue, the dorsal surface of the skull was opened and part of the brain excised. The now exposed pituitary gland and adjacent hypothalamic part of the brain were then fixed in situ for 24 hr. We used Bouin's fixative and Bouin-Hollande-sublimate. After dissection and the usual procedures of dehydration, the glands were embedded in Paraplast. Serial sections 5 or 7 μ thick were cut longitudinally.

The following staining techniques were applied to sections of tissue fixed in either of the two fixatives: Mallory-Azan, modification of Heidenhain (abbreviated Azan; Romeis, 1948); Masson's trichrome including ponceau de xylidine (abbrev. Masson; Culling, 1957, p. 294–295); Bargmann's chrome hematoxylin-phloxine (abbrev. CHP; Pearse, 1961, p. 819); Gomori's aldehyde-fuchsin (abbrev. AF; Gomori, 1950; staining solution prepared according to Elftman, 1959; methods applied with and without previous oxidation of sections in $KMnO_4$ – H_2SO_4); periodic acid-Schiff for demonstrating mucoid substances (abbrev. PAS, Culling, 1957, p. 206–207); alcian blue – PAS – orange G for further differentiation of mucoid granules (abbrev. AB – PAS; McManus and Mowry, 1960, p. 63–64).

RESULTS

General Morphological Structure

The structure of the teleost pituitary is variable and the nomenclature of its various regions is in a state of confusion (Gorbman, 1965; van Oordt, 1965). We have employed the terminology proposed by Pickford (see Pickford and Atz, 1957) for the main subdivisions of this gland. This terminology is useful for topographical description and avoids unwarranted suggestions as to functional analogies between parts of the teleost pituitary and those in pituitaries of other groups of vertebrates. The sockeye pituitary has four defined regions:

pro-adenohypophysis, meso-adenohypophysis, meta-adenohypophysis (these three together generally being considered to be homologous to the mammalian adenohypophysis), and the neurohypophysis. Their topographical relationships are best shown in median sections (Fig. 1 and 2). The smallest part, pro-adenohypophysis (also known as anterior lobe or follicular lobe) occupies a dorso rostral position. Its thin projections, which extend ventrally and laterally, cover a large part of the gland's anterior aspects. With the exception of the cells lying immediately underneath the capsule, the cells of the pro-adenohypophysis are arranged in follicles, which extend ventrally and laterally.

The meso-adenohypophysis (dorsal lobe) comprises most of the pituitary's central portion. It is relatively small in immature specimens, where it is enclosed ventrally and ventro-posteriorly by the meta-adenohypophysis, whereas its dorsal aspect is covered by the neurohypophysis. The superior part of this region consists of cell columns which penetrate slightly into the neurohypophysis. Characteristically these peripheral lobules always contain groups of undifferentiated cells.

The meta-adenohypophysis occupies the ventral half of the pituitary. In sections it is seen as a triangle composed of strands of columnar cells separated from one another by neurohypophyseal tissue.

This neurohypophysis (neural lobe, pars nervosa) surrounds the infundibular cleft and forms the attachment of the pituitary to the hypothalamus. The lobe covers most of the meso-adenohypophysis and forms a wedge between this last region and the meta-adenohypophysis. Its largest portion is found in the ventro-posterior part of the pituitary, where it ramifies between the strands of the meta-adenohypophysis.

Although we noted some capillaries between the follicles of the pro-adenohypophysis, this area contains remarkably few blood vessels. The meso-adenohypophysis is better vascularized. Both in the central parts of the cell columns and between them, small capillaries occur, and several larger sinusoids were also present. The blood supply of the meta-adenohypophysis itself appears to be poor, but numerous vessels, small and large, are present in the neurohypophyseal ramifications bordering immediately upon the cell columns of this lobe.

The foregoing reflects the situation in the sexually immature sockeye. During sexual maturation, however, considerable changes take place. The most conspicuous of these concerns the development of the meso-adenohypophysis. Due to cell multiplication as well as cell hypertrophy this lobe increases quite rapidly and eventually forms more than half of the total pituitary. In the spawning fish the meso-adenohypophysis occupies the entire dorsal half of the gland and, in addition, penetrates as a wedge between the pro- and the meta-adenohypophysis. Also, in mature fish, the histological characteristics of the various parts are no longer sharply defined and follicles from the pro-adenohypophysis, as well as cell groups belonging to the meso-adenohypophysis are occasionally found in the deeper parts of the meta-adenohypophysis.

For further detailed analysis we prefer to describe the characteristics of the pituitary and its cell types for each of the various stages of maturity. Our techniques revealed nine different cell types. Distinction was made on the basis of their tinctorial and histochemical properties. All nine cell types were found in the pituitaries of practically all immature, mature, and many of the spent fish but there is a gradual shift in the relative proportions of each type of cell during ripening of the gonads. Table II summarizes the characteristics of each cell type. These types, listed in numerical order, will, in this chapter, be referred to by their numbers rather than by terms describing their affinities for dyes, or by Greek letters. In the Discussion, functional identification and reference to analogous cell types described by other investigators will be made where it is deemed proper.

Sexually Immature Fish (Stage I)

Pro-adenohypophysis.— Most of the cells in this area were columnar and were arranged in follicles. These usually had a spherical lumen, but oval or even short tubular lumina were no exception. The material in the lumina stained weakly with aniline blue or light green. Occasionally it contained similarly staining granules. Moreover, in most cases some chromatin, often resembling a small nucleus, was present (Fig. 3).

Cell type 1.— The cytoplasm in these cells contained in its basal portion, around the peripherally located nucleus, a large number of small granules which could be stained with acid fuchsin (Masson), azocarmin (Azan), or phloxine (CHP) (Fig. 13).

Cell type 2.— These cells, which were morphologically quite similar to type 1, differed from it in that they contained a comparatively small number of fairly large granules which were distributed throughout the entire cytoplasm, were strongly PAS-positive, and also stained with aniline blue (Azan), light green (Masson), and aldehyde fuchsin (AF), especially when this last method is preceded by oxidation of the sections. Usually these cells, primarily situated in the ventral extension of the pro-adenohypophysis, were found in very limited numbers. In some fish they were entirely absent. These were invariably specimens with exceptionally low gonad weights.

Meso-adenohypophysis.—,This lobe consisted of columns of cells, separated by sparse amounts of connective tissue in which very small blood vessels occurred. The structure of these columns is best shown in the dorsal aspects, where they extend into the neurohypophysis. The deeper parts appeared as a mass of densely packed cells. Six different cell types were recognized.

Cell type 3.— These cells (Fig. 14) form small groups in the uppermost parts of the dorsal columns. They had very little chromophobe cytoplasm, small spherical nuclei, and were not unlike embryonic cells. Mitoses were frequently observed here.

Cell type 4.— In the central parts, particularly the cores of the cell columns, cells (Fig. 14) were found which differ from type 3 in that they were larger, more or less spindle-shaped, with elongated or irregular nuclei, and possessed

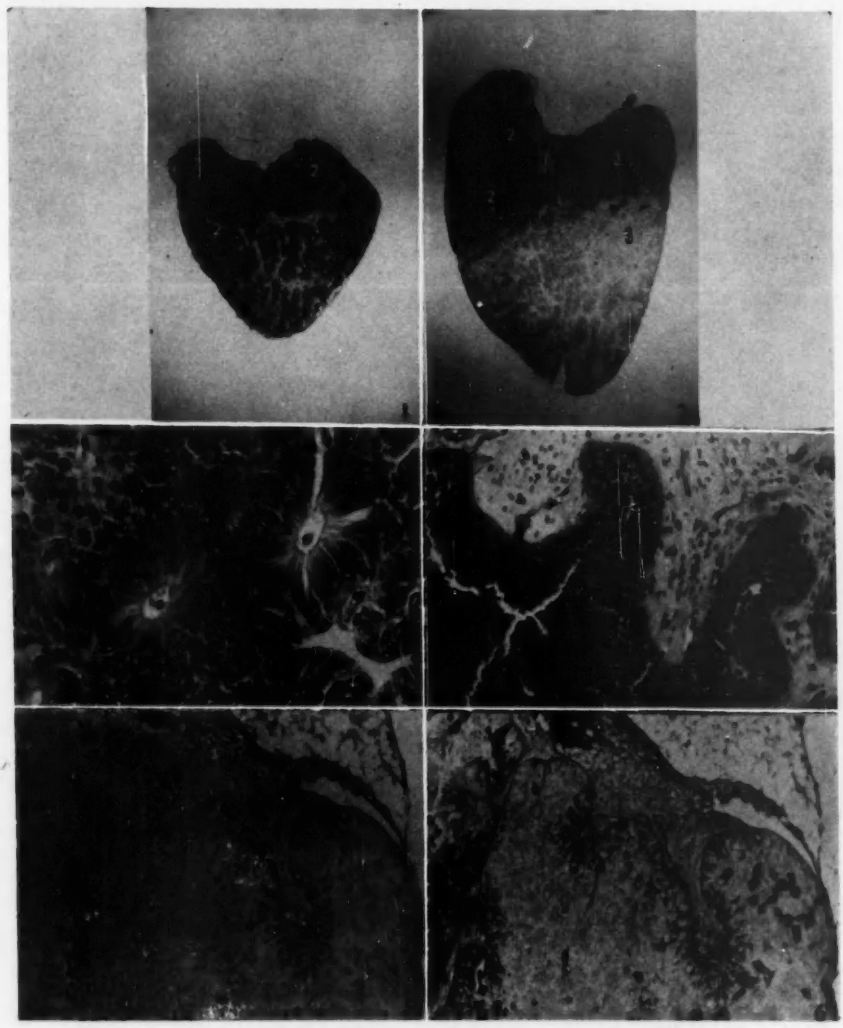

FIG. 1. Median section of pituitary gland in sexually immature sockeye (Stage I) showing: (1) pro-adenohypophysis, (2) meso-adenohypophysis, (3) meta-adenohypophysis, and (4) neurohypophysis. Note intermingling of neurohypophyseal tissue with meta-adenohypophysis. Azan. ×16.

FIG. 2. Median section of pituitary gland in sexually mature sockeye (Stage III). The various components are indicated as in Fig. 1. Note the visible increase in the size of gland, especially evident in the proportion of area (2). Also, note the compactness of the latter area. Azan. ×16.

FIG. 3 Sagittal section through pro-adenohypophysis of immature sockeye (Stage I). Two follicles are cut centrally and show finely granulated cytoplasm of the columnar cells. Note material resembling pyknotic nuclei in the lumina of the follicles. Masson. ×295.

FIG. 4. Sagittal section through pituitary of immature sockeye (Stage I) showing arrangement of palisade-like layer of cells of type 8 between pro-adenohypophysis (lower left) and neurohypophysis (light area). Masson. ×115.

FIG. 5. Section through pituitary of mature sockeye (Stage III) showing weakly stained type 8 cells bordering upon neurohypophysis. Cells of type 6 cannot be clearly distinguished. AF. ×70.

FIG. 6. Identical section as shown in Fig. 5. Section oxidized and restained with AF. Note that type 8 cells have same color intensity as in Fig. 5 but that cells of type 6 are now deeply stained. AF. ×70.

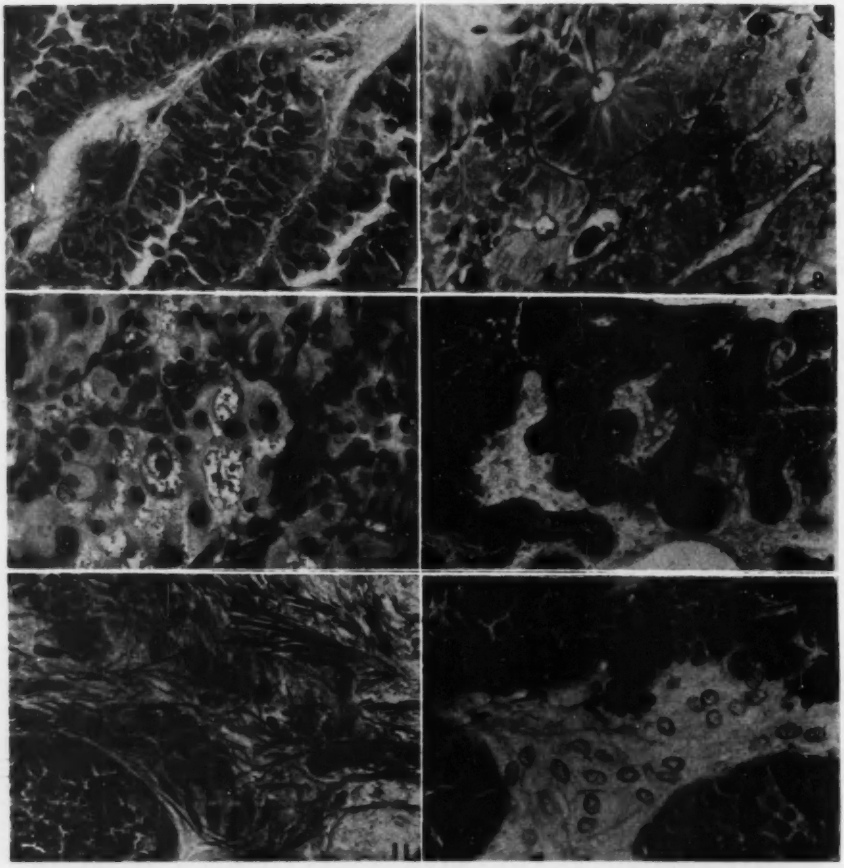

FIG. 7. Strands of cells of the meta-adenohypophysis in a sexually immature sockeye (Stage I). In many of the columnar cells acidophilic material is located closest to the surrounding membrane. Note the presence of small blood vessels in the ramifications of the neurohypophysis. Masson. ×295.

FIG. 8. Part of the pro-adenohypophysis of a sexually ripening sockeye (Stage II) showing several heavily granulated cells of type 2. Compare with Fig. 3. PAS. ×295.

FIG. 9. Part of the meso-adenohypophysis of a sexually mature sockeye (Stage III) containing many type 7 cells in various stages of cytoplasmic disintegration. Masson. ×295.

FIG. 10. Disruption of cell strands in the meta-adenohypophysis of a sexually mature sockeye (Stage III). Note the abundance of chromatin-poor nuclei in the neurohypophysis. CHP. ×70.

FIG. 11. Part of the neurohypophysis of a sexually mature sockeye (Stage III) near the dorsal cell columns of the meso-adenohypophysis (shown lower left). Note abundance of fibroblasts (lower center) and dense concentration of connective tissue fibers. Masson. ×295.

FIG. 12. Dorsal part of neurohypophysis in spent sockeye (Stage IV). Membrane surrounding the cell column disrupted. Note migration of undifferentiated cells (type 3) into the neurohypophysis. Masson. ×295.

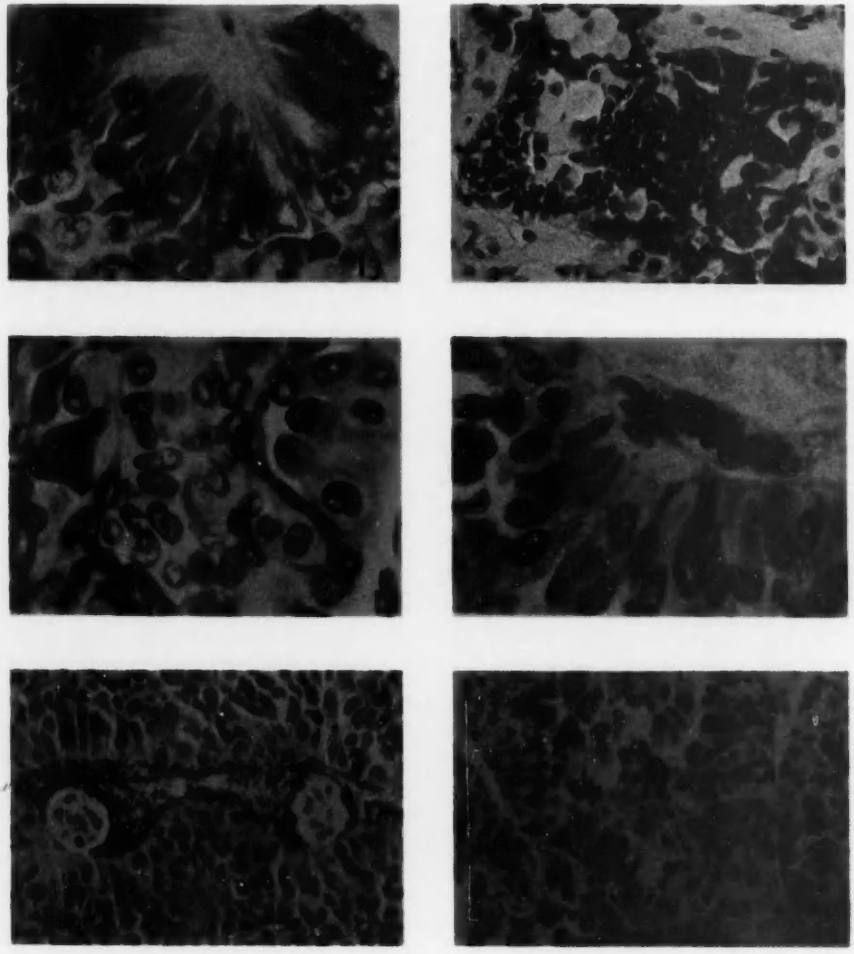

FIG. 13. Part of pro-adenohypophysis in sexually immature sockeye (Stage I) showing follicle cells (type I) loaded with fuchsinophilic granules. Note the presence of a single cell of type 2 containing only relatively few coarse granules. Masson. ×705.

FIG. 14. Dorsal part of the neuro-adenohypophysis in sockeye (Stage I). Note cells of type 3 (far left, bordering upon neurohypophysis), type 4 (center portion), type 5 (stained red), type 7 (pale cells). Masson. ×295.

FIG. 15. Same area as shown in Fig. 14 showing large cells of type 5 (red), type 6 (small cells with coarse green granules), and type 8 (orange–brown) Masson. ×705.

FIG. 16. Cells of type 9 in meta-adenohypophysis of sexually immature sockeye (Stage I)• Note concentration of fuchsinophilic granules near blood vessel in the neurohypophysis. Masson. ×705.

FIG. 17. Accumulation of neurosecretory substances around small blood vessels in ramifications of neurohypophysis between tissue of the meta-adenohypophysis. CHP. ×295.

FIG. 18. Part of the meso-adenohypophysis of a sexually ripening sockeye. Note abundance of type 6 cells containing PAS positive granules. PAS. ×295.

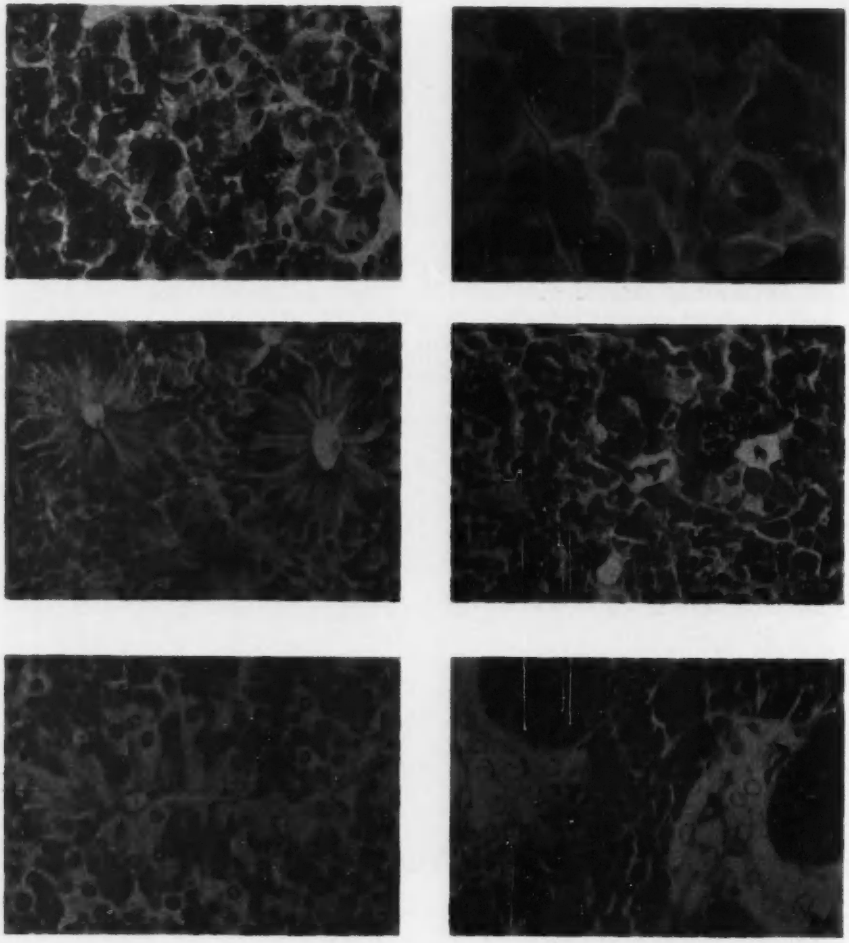

FIG. 19. Part of the meso-adenohypophysis of a nearly sexually mature sockeye, showing cells type 5 (red) and type 6 (dark blue). Note that in the latter cells only the smaller granules are stained. Compare with Fig. 20. CHP. ×295.

FIG. 20. Meso-adenohypophysis of sexually mature sockeye (Stage III) showing cell type 6. Note that alcian blue stains most of the cytoplasm including the small granules whereas only the large granules are PAS positive. AB – PAS ×705.

FIG. 21. Section through pro-adenohypophysis of sexually mature sockeye (Stage III), showing cell types 1 and 2. Note that cell type 2 (granulated) is much larger than cell type 1. The cytoplasm of the latter is practically devoid of granules but their apical ends are deeply stained. Masson. ×295.

FIG. 22. Rostro-ventral part of meso-adenohypophysis in sexually mature sockeye (Stage III). In this area cells of type 6 are shrunken, lack regular granulation, and have pyknotic nuclei. Masson. ×295.

FIG. 23. Dorsal column of meso-adenohypophysis in sexually mature sockeye (Stage III). Note that cells of type 6 are swollen, their cytoplasm is practically insensitive to aniline blue, and their nuclei are relatively poor in chromatin. Azan. ×295.

FIG. 24. Section through pituitary gland of sexually mature sockeye (Stage III) showing part of the complex of meta- and neurohypophysis. Note shrinkage of many cells, absence of fuchsinophilia in their cytoplasm, and absence of sharp border between cell columns and adjacent parts of the neurohypophysis. The latter contains many characteristic large chromatin-poor nuclei (ghost cells). Masson. ×295

TABLE II. Staining reaction of cytoplasmic granules in secretory cell types of the sockeye adenohypophysis.

Cell types	1	2 and 6	5	7	8	9
Particulars of granules	Fine granules	Fine to coarse granules	Fine granules	No distinct granules but cytoplasm as a whole appears granular		No distinct granules; staining properties refer to apical portion of cytoplasm
Heidenhain's Azan	Red to orange	Blue	Red	–	Lavender	Blue
Masson trichrome	Red	Green	Magenta	–	Orange–brown	Red
Gomori's aldehyde-fuchsin, without previous oxidation	–	Faintly purple	–	–	Faintly purple	Purple
Gomori's aldehyde-fuchsin, after oxidation	–	Deep purple	–	–	Faintly purple	–
Bargmann's chrome-hematoxyline-phloxine	Brick red	Blue–black[a]	Brick red	–	Grey	–
PAS, counterstained with tartrazine	Yellow	Purplish red[b]	Yellow	Pale pink	–	–
Alcian blue–PAS counterstained with orange G	Yellow–orange	Blue, purple, & red[c]	Yellow–orange	Pink–orange	–	Yellow

[a]Usually only the smaller granules could be demonstrated. The location of the large granules was indicated by spherical unstained areas.
[b]The larger granules were red.
[c]The background and the very small granules were blue, the medium-sized ones purple; only the largest granules stained red.

cytoplasm with weak affinities for both acid and basic dyes. In most specimens this cell type was fairly abundant.

Cell type 5.— This cell type (Fig. 14 and 15) was easily recognized because of the acidophilic granules which are spread throughout the entire cytoplasm. These cells were variable in shape and size, and had a nucleus which was always indented and frequently appeared sickle-shaped. Although present in all parts of the lobe, they were most abundant in the lateral parts, and in the area close to the pro-adenohypophysis.

Cell type 6.— Apart from their size and shape, which resembled those of type 5, this category (Fig. 15) was identical to the cells of type 2 in the pro-adenohypophysis. In most specimens their occurrence was sparse. Quite generally their abundance and that of the type 2 cells were correlative. They seem to first develop in the vicinity of the ventral extension of the pro-adenohypophysis.

Cell type 7.— The dorsal columns contained a number of conspicuously large cells with spherical nuclei (Fig. 14). Their finely granulated cytoplasm was refractory to most dyes, but assumes a slightly pinkish colour with the PAS-technique. In many specimens the cytoplasm showed signs of vacuolar disintegration. In advanced stages these vacuoles contained distinct acidophilic material, most likely cytoplasmic debris. Although the loss of cytoplasm was often considerable, nuclear degeneration was rarely observed.

Cell type 8.— A layer of regularly arranged columnar cells (Fig. 4) formed the dorso-rostral aspect of the meso-adenohypophysis. The cells somewhat resembled those of the follicular area, but their organization and tinctorial properties differed. This cell layer often formed the dorsal aspect of the pro-adenohypophysis and extended between the pro- and the meso-adenohypophysis over some length, but was at least in part separated from the latter by neurohypophyseal tissue. In addition, small, isolated cell groups having the same staining properties, were found in close proximity, scattered in the neurohypophyseal tissue. The cells of type 8 are rather insensitive to dyes, but could be readily recognized in Masson stained sections, where their very finely granulated cytoplasm assumed a distinct orange–brown colour (Fig. 15). Also, they reacted weakly to the aldehyde fuchsin technique. The results of the latter technique were not affected by previous oxidation of the sections (cf. Fig. 5 and 6).

Meta-adenohypophysis.— The strands of cells in this region were separated from the ramifications of the neurohypophysis by a delicate membrane. The peripheral cells were oriented perpendicular to this membrane; their nuclei were usually located away from the membrane (Fig. 7).

Cell type 9.— In this area only one cell type could be distinguished. Although, in general, the cytoplasm of these cells showed no pronounced affinity for any of the dyes used in this investigation, in many fish the slender parts of the cells near the surrounding membrance could be stained with acid fuchsin (Masson), aniline blue (Azan), and weakly with phloxine (CHP) (Fig. 16).

Neurohypophysis.— The bulk of the material in this lobe appeared to consist of fibres. A small fraction of these were collagenous fibres and we assume the remainder to be nervous tissue. The techniques employed do not differentiate between true nerve axons and neuroglia. The neural lobe contained comparatively few cells. Some were fibroblasts with elongated nuclei. Of the others, only oval nuclei could be seen, the cytoplasm being indistinguishable. Occasionally, large, irregular amounts of colloid were noted in the dorsal parts of this lobe. In addition, large amounts of neurosecretory material were present, particularly in the ventral ramifications. This material consisted of numerous small droplets, which could be stained distinctly with either the CHP- or the AF-method. Accumulation of this material was seen in the more extensively vascularized areas in the immediate vicinity of the meta-adenohypophysis (Fig. 17).

Cell division was noted in each of the three lobes of the adenohypophysis. We have no quantitative data, but mitotic activity was seen most frequently in the upper parts of the columns in the meso-adenohypophysis as well as in all parts of the meta-adenohypophysis. To a lesser extent, mitotic figures were also seen in the follicle cells of the pro-adenohypophysis and in the deeper parts of the dorsal lobe. No mitosis was observed in the neurohypophysis.

Fish with Maturing Gonads (Stage II)

Pro-adenohypophysis.— Little change was noted in the structure of this lobe. It seems to have extended in the ventral direction. Both the follicles and their lumina were somewhat larger. The position of the granules in the tall cells of type 1 had shifted towards the cell apex. In many cells, moreover, granules could no longer be discerned but in these cells the apical part of the cytoplasm was strongly acidophil. Also the adjacent parts of the colloid stained deeply with acid dyes. The cells of type 2 were more abundant (Fig. 8), particularly in the ventral portion. They were distended and contained a large number of relatively coarse PAS-positive granules.

Meso-adenohypophysis.— This lobe had expanded in a dorso-posterior as well as a ventro-rostral direction. The latter area formed a wedge between the pro- and the meta-adenohypophysis. Cells of type 3, now somewhat decreased in number, had retained their position in the upper parts of the dorsal cell columns. Cells of the second undifferentiated type (type 4) were also reduced in number. In contrast the acidophils, (type 5) were more abundant, especially in the dorsal regions. This was at least the case in the material of 1965; the samples of 1963 and 1964 showed generally fewer of these cells, with little increase in their numbers even during the later stages of maturation. The main change in the meso-adenohypophysis concerned the cells of type 6. Towards the end of sexual maturation these made up more than 50% of the entire lobe and were particularly numerous along the boundaries with the pro- and the meta-adenohypophysis. The cells were enlarged and, like the type 2 cells, contained many granules which showed a strong affinity for aniline blue (Azan) or light green (Masson) and were PAS-positive (Fig. 18). Chrome-hematoxyline

usually showed only the smaller granules, leaving spherical areas which indicated the location of large granules (Fig. 19). Possible chemical differences between the granules of any one of these cells was further indicated by the results of the Alcian blue–PAS technique, which stained the smallest granules deep blue, the medium-sized ones purple, and the largest ones red (Fig. 20). Chromophobe cells of type 7 were present in all pituitaries of this category of fish, although their number was now reduced and, with few exceptions, their cytoplasm was in an advanced state of vacuolar degeneration. No changes could be seen in the cells of type 8.

Meta-adenohypophysis and neurohypophysis.— Of the complex of these two parts, now occupying the ventral triangular part of the pituitary, the first had proportionately increased. The strands of cells were wider and in many cases somewhat disrupted internally. Also the membrane indicating the boundary between these strands and the neurohypophysis was less distinct and occasionally seemed to be interrupted. In a few cases cells were found migrating into the neural lobe, which probably explains the apparent increase in the number of nuclei observed in the latter tissue. Few mitoses were found and the cytoplasm in some cells was vacuolated, whereas in others the cytoplasm and the nucleus were shrunken. Signs of focal degeneration were, however, not widespread and most of the tissue showed the same characteristic structure as seen in the sexually immature specimens. Also, the acidophilia of the cytoplasm, although less pronounced and generally more evenly distributed throughout the whole cell, was still present.

The neurohypophysis showed a denser pattern of fibers and possessed more connective tissue. Also several large open spaces were noted. The number of fibroblast nuclei had increased. Especially in the ventral ramifications, a number of atypical nuclei were seen, which were poor in chromatin and lacked nucleoli. No significant change in the amount or distribution of neurosecretory material could be detected.

Mature Fish (Stage III)

Mainly due to further development of the meso-adenohypophysis, the pituitary had now reached its largest size (Fig. 2). The following description is based on 44 mature, apparently healthy specimens, killed no later than January 15. The latter date marks approximately the end of spawning for these fish in their natural environment.

Pro-adenohypophysis.— With the exception of most of the specimens collected during 1965, the cells of type 1 were devoid of granules. In all fish, however, the apical portions of their cytoplasm and the adjacent parts of the follicle content exhibited a characteristic acidophilia (Fig. 21).

Cells of type 2 were numerous, very large, and full of PAS-positive granules. Particularly those in the ventral follicles were bulky, pushing the adjacent slender acidophils aside (Fig. 21). Degenerative features such as pyknosis of nuclei, loss of cytoplasm, and cyst-formation in this lobe were seen occasionally.

Meso-adenohypophysis.— As in the pro-adenohypophysis, the greatest changes in the structure of the meso-adenohypophysis took place during this period of sexual development. Although undifferentiated cells of type 3 were noted in all specimens studied, a distinct decrease in their number was evident. An even greater reduction was seen in the number of cell type 4: indeed this type was frequently absent. On the other hand, the acidophils (type 5) were more numerous, particularly in the dorsal columns (Fig. 19). However, except for the increase in their number, these latter cells seemed to have undergone little or no change. Cells of type 6 were very abundant (Fig. 19) and found in all parts of the lobe. Moreover, these cells exhibited changes which suggest two different kinds of degeneration. The cells in the deeper parts of the gland, in particular the ones adjacent to the ventral projection of the pro-adenohypophysis, often showed shrinkage. In these shrunken cells, granules could no longer be discerned and the nuclei showed various stages of pyknosis. Their cytoplasm had assumed the tinctorial and histochemical properties formerly exhibited by their granules and it was stained intensively (Fig. 22).

Another form of degeneration was seen in the more dorsally located groups of type 6 cells. Here, the cells became devoid of granules and the cell content showed a reduced affinity for dyes (Fig. 23). Their nuclei never underwent pyknosis and showed a reduction of stainable chromatin. In advanced stage of degeneration, these nuclei were surrounded by only some shreds of cytoplasm. In many fish only a fraction of the cells were affected, but occasionally such degenerative features were found to be widespread.

Chromophobe cells (type 7) were recognized in only 9 out of the 44 mature specimens studied. The cytoplasm of these cells was in an advanced stage of disintegration (Fig. 9). Where both these cells and those of type 6 were degeneratively affected it was difficult to differentiate between them. For this reason chromophobes could not be recognized with certainty in 16 specimens of this group. In the remaining 19 fish which all had intact cells of type 6, chromophobes were found to be practically absent.

Cells of type 8 did not show appreciable differences from those seen in earlier stages of sexual development. In cases where the degenerative phenomena, described above, were pronounced, they were reflected in disruption of whole cell columns in the meso-adenohypophysis.

Meta-adenohypophysis and neurohypophysis.— The changes which took place during stage II of sexual maturation were generally accentuated. Almost all of the cells had now lost their affinity for dyes. Furthermore, a variable degree of disintegration of the cells strands was evident (Fig. 10). However, although in many of the 44 specimens studied focal disruption of the cell columns and of the membrane, pyknosis of nuclei, and loss of cytoplasm were seen, in only six fish were these changes advanced and did they include considerable cytolysis and migration of cell nuclei into the neurohypophysis. In three others these features had developed into extensive degeneration which was also present in other parts of the pituitary. There was a definite increase in the amount of connective tissue between the cell strands of the meta-adenohypophysis (Fig. 11).

No appreciable changes were observed in the amount or the distribution of neurosecretory material. Here too, considerable individual variation was noted. In the neurohypophysis the conspicuous cells, characterized by chromatin-poor nuclei, and unstainable cytoplasm, (termed "ghost cells" by Robertson and Wexler, 1962b), had increased in number (Fig. 24). There is evidently a positive correlation between their abundance and the degree of disintegration of cell groups in the meta-adenohypophysis.

Although in this stage mitotic activity was markedly reduced, as compared with stages I and II, occasional cell divisions were noted in all parts of the adenohypophysis.

Spent Salmon (Stage IV)

This description results from a study of 44 apparently healthy specimens with the exception of all fish which were kept for an unusual time before they were allowed to spawn (later than January 15) or those that were kept for more than 3 weeks after completion of spawning.

The general structure of the pituitary did not differ from that in the previous stage, but the degenerative characteristics were somewhat more pronounced, particularly in the meso-adenohypophysis where small groups of undifferentiated cells were seen migrating into the neurohypophysis (Fig. 12).

Pro-adenohypophysis.— This part differed from that in the previous group only in that several of the large type 2 cells were slightly degenerative and occasionally showed pyknotic nuclei. Loss of granules and vacuolization of the cytoplasm was noted frequently.

Meso-adenohypophysis.— Cells of type 3 showed a further reduction in number although small numbers were present in most specimens, whereas type 4 was now absent. The acidophils (type 5) were abundant, often large, but did not show signs of disintegration, except in cases where entire cell-columns were affected. In many specimens the cells of type 6 had undergone further degranulation and vacuolization. Disintegrated chromophobe cells (type 7) were recognized in 15 out of 44 fish, absent in 16 others, and in the remaining 13, they could not be recognized with certainty because the type 6 cells were also in an advanced stage of deterioration. The characteristics of cell type 8 were not different from those in the mature unspent fish.

Meta-adenohypophysis and neurohypophysis.— This complex showed only slight differences when compared with unspent mature fish. Focal deterioration was recognized in most of the 44 specimens examined but advanced stages could be seen in 6 fish only and extensive degeneration was present in 6 others.

DISCUSSION

The morphological structure of the sockeye pituitary is essentially the same as that of the spring salmon (Robertson and Wexler, 1962b), the rainbow trout (Robertson and Wexler, 1962a), and the Atlantic salmon (Olivereau, 1954). However, there appear to be differences in the cellular composition as well as in the manifestations of pituitary activity during the period of sexual ripening and spawning.

Our data indicate the existence of nine different cell types. From their description in the previous sections, it will be evident that these cell types do not necessarily reflect nine different functions with regard to the production of pituitary hormones. On the basis of changes in cell structure and in the relative proportions of each type during sexual maturation, we are led to believe that the undifferentiated cell types 3 and 4 in the meso-adenohypophysis represent stages in the development of the other cells in this region. Cells of type 4, present mainly in immature specimens, most likely develop into type 6 and perhaps also into type 5. On the other hand, cells of type 3, resembling embryonic cells and present in fish of all stages, be it in amounts progressively decreasing with maturation, are probably a general source of all other cell types in the meso-adenohypophysis.

Furthermore, it may well be that cell types formed in different lobes of the pituitary are functionally identical. For instance, the PAS-positive cells in the pro-adenohypophysis (type 2) and those in the meso-adenohypophysis (type 6) show a striking similarity, not only in their tinctorial and histochemical properties (Table II), but also with regard to their activity during maturation. Thus, considering cellular characteristics and morphological aspects of activity, our material suggests the existence of six functionally different cell types in the sockeye adeno-hypophysis: (*a*) type 1; (*b*) type 2 and 6; (*c*) type 5; (*d*) type 7; (*e*) type 8; (*f*) type 9.

Our observations with regard to cell type 1, which forms the dominant cell in the pro-adenohypophysis, confirm those of Robertson and Wexler (1962b). However, our results were somewhat inconsistent. Whereas degranulation of the follicle cells was noted during maturation in the fish of 1963 and 1964, these cells underwent very little change in the sockeye of 1965, where, even in spent fish, acidophilic granules were still present. It is interesting that, although the granulation could be seen in all sexually immature fish captured in freshwater, it was entirely absent in five specimens obtained from salt water (Barkley Sound) a few days before they would normally have entered the freshwater phase of their migration. It is worthwhile to mention that Olivereau (1954) reported an increased secretory activity of these cells in Atlantic salmon as soon as they enter freshwater. Olivereau and Ball (1964) presented evidence that erythrosinophil cells in comparable areas in pituitaries of two other teleosts (Genus *Poecilia*) show increased activity upon transfer of the fish from seawater to freshwater. Their investigations indicate, furthermore, that in these fish prolactin is involved in the adaptation to osmotic changes, and they postulated that this hormone originates from the erythrosinophil cells (Ball and Olivereau, 1964; Ball, 1965). Further studies on the functional identification of the acidophil cells in the pro-adenohypophysis of the sockeye are in progress.

The occurrence of a second cell type in the follicular epithelium, which we described as type 2, has been reported previously by Olivereau (1954) for the Atlantic salmon. Robertson and Wexler (1962a) also found these cells in the rainbow trout, but did not mention them in their study of Pacific salmon (Robertson and Wexler, 1962b). In view of the similarities between these cells and those of type 6, their possible function will be described hereafter.

Our cell types 5, 6, and 7, all in the meso-adenohypophysis, most likely correspond with the acidophil, basophil, and chromophobe cells respectively, described in previous studies of the pituitary of salmonids. Of these cell types, the first (type 5) seems to undergo few changes in the period of sexual ripening. The abundance of these cells shows individual variation as well as differences between fish of different years. Their numbers were largest in fish of the 1965 run. Olivereau (1954) reported little change in the relative numbers of these cells in Atlantic salmon during migration and spawning, but a marked increase in cytological activity. Our results, although not quantitative, indicate a definite increase in the relative proportion of these cells as well as a general increase in their size. This confirms the observations by Robertson and Wexler (1962b) on chinook salmon. However, unlike the latter investigators, we have seen very few signs of degeneration in these cells, even in spent fish. Olivereau suggested that in species of *Oncorhynchus*, and a few other teleosts, these cells produce growth hormone (Olivereau, 1963; Olivereau and Ball, 1964, 1966). It seems to us, however, that this concept stems mainly from studies of a similar cell type in the pituitary of other vertebrates, but, so far, lacks direct experimental proof.

Our sockeye material enabled us to follow a complete cycle of activity in the cells of type 6, generally referred to in the literature as basophils. They appear when the fish are about to enter, or have just entered, freshwater and develop progressively during further maturation. This parallels the development of the cells of type 2, in the pro-adenohypophysis, which show the same tinctorial properties and are also PAS-positive. They differ somewhat in that the latter show little loss of cytoplasm and pyknosis, even in fully mature or spent fish, phenomena which are quite common and occasionally widespread in the PAS-positive cells of the meso-adenohypophysis at these stages. As stated before, these two categories of cells may well have an identical function. Most likely they produce gonadotrophic hormones, the synthesis of which in teleosts has been generally linked to a basophil cell type in the meso-adenohypophysis (Pickford and Atz, 1957).

It has recently been suggested that in some teleosts two cell types participate in gonadotrophic activity. This seems to be the case in the eel (Olivereau and Herlant, 1960; Olivereau, 1961, 1963), the chinook salmon (Olivereau and Ridgway, 1962; Olivereau, 1963), and the mullet, *Mugil cephalus* (Stahl, 1963; Leray and Carlon, 1963). It is interesting to note that of these two types of gonadotrophic cells, described for the chinook salmon as *beta* and *gamma* cells (Olivereau, 1963), the first one is obviously identical to our type 6, and the *gamma* cell resembles our cell type 7 in an advanced stage of vacuolar degeneration. It seems difficult at this stage of our investigations to functionally identify this last chromophobe cell type. Robertson and Wexler (1962b), in their study of the pituitary of the chinook salmon, consider them to be exhausted basophils, an opinion tentatively suggested earlier by Olivereau in her account of the pituitary gland of the Atlantic salmon (Olivereau, 1954). Our findings in the sockeye do not support this. First, intact chromophobes appear to be most

abundant in sexually immature fish. At this time "basophils" are either absent or, if present, are small and relatively few in number. Also, the chromophobes undergo degeneration and have indeed largely disappeared at the time when the first depleted basophils can be seen. Moreover, disintegration of the chromophobes always seems to start with the development of one of two large vacuoles in their cytoplasm, whereas in the cells of type 6, degeneration is usually preceded by loss of granules and can often be recognized by shrinkage and pyknosis of the nuclei. Although the changes in activity of these two categories of cells in the sockeye are separated in time, they both take place during gonad development. The possibility that cell type 7 has a function related to this process can therefore not be excluded.

In this connection, it is of interest that the period of gonad maturation in Pacific salmon is further characterized by a considerable hypertrophy of the adrenocortical tissue. This was first reported by Robertson and Wexler (1957, 1959, 1960) and Hane and Robertson (1959) for chinook salmon, an observation which we have confirmed in our sockeye (van Overbeeke, 1967). So far we have been unable to correlate this phenomenon to the changes in cell type 7, or any other cell type in the pituitary.

The occurrence of a palisade-like layer of cells (type 8), bordering upon the neurohypophysis and partly separating the meso-adenohypophysis rostrally from the pro-adenohypophysis, has not, to our knowledge, been described earlier in studies of the pituitary of salmonids. However, cells of a similar type and comparable location, called X-cells, were reported previously by Olivereau (1963) for the eel, *Anguilla anguilla*. Recent experimental investigations with *Anguilla anguilla*, *Poecilia* (*Mollienisia*) *latipinna*, and *Poecilia* (*Mollienisia*) *formosa*, where these cells were referred to as *epsilon* cells, suggest that they are the source of corticotrophic hormone (Olivereau and Ball, 1963, 1964, 1966; Olivereau, 1965; Ball et al., 1965; Ball and Olivereau, 1966). If, in the sockeye, these cells have a corticotrophin-producing function, one would expect to see evidence of changes in their activity, in view of the hypertrophy of the adrenocortical tissue, which we noted in our fish. However, no changes were seen in the cells of type 8, which could be correlated to the last phenomenon.

Meta-adenohypophysis and Neurohypophysis

The complex of these two lobes is usually associated with chromatophore concentrating and dispersing hormones (intermedin). However, there is little information on where these hormones are produced and stored (see Pickford and Atz, 1957).

In salmonids the cells of the meta-adenohypophysis do not show distinct tinctorial properties, but are generally described as weakly acidophil (Olivereau, 1954; Robertson and Wexler, 1962a, b). Our findings in the sockeye confirm the general observation that the cells are uniform. Olivereau (1954) reported definite secretory activity in these cells in Atlantic salmon only during the early stages of their migration in freshwater. She noticed elaboration of acidophil products into the adjacent parts of the neurohypopyseal tissue. In the

sockeye, the only signs indicating the secretory function of these cells (type 9) was loss of acidophilia, particularly in the peripheral parts of their cytoplasm towards the end of maturation. As far as we know, there are no data in the literature which suggest the function of the meta-adenohypophysis in Pacific salmon.

The most conspicuous non-cellular component of the neurohypophysis was the neurosecretory substance, revealed by the chrome-hematoxylin and aldehyde-fuchsin techniques. In some of our specimens we were able to examine the hypothalamus and its connections with the pituitary and there can be little doubt that the neurosecretory material originates in the cells of the nucleus preopticus. The hypothesis that in teleosts this product is concerned in osmoregulation, finds considerable support in various investigations on the effect of changing salinities, particularly in euryhaline species, although the results are somewhat inconsistent (Arvy and Gabe, 1954; Arvy et al., 1954; Fridberg and Olsson, 1959; Korn, 1960; Schiebler and Hartmann, 1963). Moreover, it should also be said that the relation between this histologically demonstrable substance and the actual hormones of the neurohypophysis is not precisely known.

In their study of the Atlantic salmon, Arvy et al. (1955) observed accumulation of neurosecretory material in the cells of the nucleus preopticus during the freshwater phase of its spawning migration along with a decrease in transportation of the products towards the neurohypophysis. The latter was found to be practically depleted in fish taken from the spawning grounds. No pertinent data have, to our knowledge, been published on Pacific salmon.

Our own data concerning this aspect are difficult to interpret. In fish captured at the entrance to Great Central Lake (which had presumably spent several days in freshwater), the amount of neurosecretory products in the neurohypophysis was higher than in any of the five specimens obtained from salt water (Barkley Sound). The amounts found in the pituitaries of mature and spent fish taken from the spawning beds in Great Central Lake later were variable but, in general, somewhat lower and, moreover, confined to the ventral ramifications of the neural lobe. In contrast, sockeye from the spawning beds in the Genesee River generally showed very large quantities of neurosecretory substance, both in unspent and spent specimens. In sockeye kept under laboratory conditions for various lengths of time, there was considerable variation and no definite trend could be determined. It is possible that in these last fish the neurosecretory activity was affected by the saltwater treatment, given as a prophylactic treatment for fungus infection.

Degeneration

It is evident that degenerative changes occur in the pituitary of both Atlantic and Pacific salmon during sexual maturation. Olivereau (1954) reported disintegration of follicles in the pro-adenohypophysis in Atlantic salmon after the fish had entered freshwater. She considers this a form of holocrine secretion, however, and noted that it was followed, later on, by

increased mitotic activity of the cells and reorganization of this lobe. Similarly, the considerable cellular destruction in central parts of the meta-adenohypophysis, accompanied by elaboration of colloid in the adjacent parts of the neurohypophysis noted during migration, was followed by restorative activity at the time of spawning, particularly in females. Olivereau regards these deteriorative alterations, at least in part, as the depleted stage in the cycle of secretory activity of the gland. Evidence that these changes do not necessarily have a bearing upon the survival of the fish may be found in her examination of several spent salmon (kelts) which were on their way back to the ocean. These fish had reassumed their silvery colour and their pituitaries showed considerable regeneration. In the spring salmon the degenerative changes in the meso- and meta-adenohypophysis are similar to those in Atlantic salmon (Robertson and Wexler, 1962b) but here this phenomenon is reported to involve actual cytolysis leading to extensive degeneration of large sections of the pituitary. No mention is made of restorative activity.

Qualitatively, our findings are similar to those in the spring salmon. In the sockeye too, disintegration of cell strands in the meta-adenohypophysis is accompanied by migration of cells into adjacent parts of the neurohypophysis where they probably develop into the characteristic "ghost cells." Occasionally this was also seen in parts of the meso-adenohypophysis. However, whereas in spring salmon Robertson and Wexler (1962b) found degenerative changes to commence already 2 months before sexual maturity, in the sockeye they usually did not develop before the fish were ready to spawn. Moreover, in the sockeye this phenomenon was by no means common. Out of 44 mature fish, 6 showed advanced degeneration, and in three other specimens only was it very extensive and had led to considerable destruction of large parts of the pituitary. For 44 spent fish these figures were 6 and 6 respectively. We did not find significant differences in this respect between males and females, or between fish which had completed spawning in their natural environment and those spawned in captivity.

The cause of degenerative processes in the pituitary as well as in other organs in mature and spawning salmonids has been related by Robertson and his collaborators to the abnormally high concentration of 17-hydroxycorticosteroids in the blood (Robertson and Wexler, 1962b; Robertson et al., 1961b, 1963) It is interesting to note that Fagerlund (1967) found the levels of cortisol in mature and spent sockeye salmon to be much lower. This might explain our observation that the degenerative alterations in this species were only moderate.

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NOTES

A Pumping System for Sampling Small Plankton

A new seawater pumping system and deck-mounted plankton collecting unit are described in this report, and results are presented of a short series of comparative pumped- and netted-plankton samples. The gear was developed to obtain samples for studies (Beers and Stewart, 1967) of the species composition and determinations of the volumes and biomass of the smallest planktonic animal forms in the marine food chain. These are missed by the routinely employed means of zooplankton sampling where relatively large mesh (200–>500 μ) nets are used.

Capabilities of our system include: (1) a delivery rate of approximately 150 liters/min (40 gal/min); (2) pumping while being towed at any level in the upper 100 m with a vessel cruising at speeds of 2–4 knots; and (3) preliminary size sorting of the plankton sample. The first two capabilities permit one to filter a relatively large volume of water at depths from 100 m to the surface in a reasonable length of time while traversing a distance of several miles. This gives an integrated sample over a relatively great distance and avoids some of the sampling problems associated with the non-random distribution of plankters. In addition to providing information on the size distribution of the plankton, the third capability alleviates the problems of clogging which are inherent in all net samplers designed to retain phytoplankton and the smaller zooplankton whenever relatively large volumes of water are sampled.

Pumping system and plankton collecting unit. — The complete system (Fig. 1) consists of: (1) a submersible pump with attached depth sensor and weight coupled at the end of (2) a length of vinyl hose incorporating strength members and electrical power leads, (3) a winch and hose-storage drum with a hydraulically operated dual chain drive, (4) a fairlead, and (5) a deck-mounted plankton collecting unit with a water meter and nylon cloth filters of various mesh size. The pump, hose, and winch drum were obtained from the marine instrumentation firm CM^2 of Mountain View, California. Whaley Engineering Company of San Diego constructed the hydraulic drive for the winch. The collecting unit was fabricated by the Plastik Arts Company of San Diego. The gear was according to specifications provided by the authors.

The pump (Fig. 1, No. 1), a six-stage centrifugal pump, is activated by a two horsepower, 440 v a-c, water-cooled motor (1750 rpm). All internal parts are of plastic and have smooth surfaces. The pump is fitted with a fin-stabilized, swivelling nozzle which should assist the horizontal intake (2 inch diam) to face forward when being towed. Pumping capacity is approximately 150 liters of seawater per minute with the pump at a depth of 200 m. A control unit for the pump motor, consisting of an input circuit breaker, an on–off

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FIG. 1. The pumping system, shown on board the R.V. *Horizon*, Scripps Institution of Oceanography, University of California. 1, submersible pump; 2, depth transducer in case; 3, weight, 4, the hose; 5, winch-hose storage drum; 6, hydraulic controls for winch-hose storage drum; 7, the fairlead; and 8, the plankton collecting unit.

switch, a variable 3-phase autotransformer for adjusting pump voltage, and an ammeter, is mounted on deck or in the ship's laboratory.

A depth transducer (Pace Engineering Co., Model CP 52 GR-150 PSIG-1233 Output Pressure Transducer), operated by a 32.4 v d-c battery power supply and mounted in a water-tight housing, is shackled below the pump (Fig. 1, No. 2). This provides a continuous monitoring of depth while the pump is being operated.

Weights for maintaining the pump in position at depths while the vessel is underway are attached at the base of the system (Fig. 1, No. 3). Routinely, a 900-lb lead-filled oxygen bottle, approximately 18 inches long and 12 inches diam, is used during pumping operations while the vessel is underway. For stationary work considerably less weight is required to maintain the pump in a vertical position.

The hose (Fig. 1, No. 4) is a 1.5-inch I.D. vinyl tube encased in a spun rayon sheath for added strength. Bound to the outer surface of the hose is a

band of polyurethane in which are embedded two $\frac{5}{32}$-inch (3000 lb rating) and one $\frac{1}{8}$-inch (1900 lb rating) galvanized steel supporting cables terminating on the pump housing. The polyurethane band also contains electrical leads (3-16 gauge wires) to supply power to the pump motor and 3-20 gauge conductors to carry signals from the depth sensor to a voltmeter on deck. An outer covering of polyurethane protects the entire hose assembly and assists streamlining. The 200 m of hose was found to be adequate for sampling through a 100 m euphotic zone at vessel speeds of 3–4 knots when using a 900-lb weight. The volume of this length of hose is 225 liters and, thus, at a pumping rate of 150 liters/min, the time for the passage of water through the hose is approximately 1.5 min.

Hose can be reeled in or out from the winch-hose storage drum (Fig. 1, No. 5) at variable speeds up to approximately 12 m/min. Removable wooden separators, "hat sections," are used on the storage drum to provide independent support for the layers of hose. A rotary water joint at the hose outlet allows for continuous pumping while the hose is being reeled in or out.

The fairlead (Fig. 1, No. 7), which guides the hose into the water, can be modified to be either deck mounted or suspended overhead from a boom or stanchion.

The plankton collecting unit consists of four circular acrylic plastic units (Fig. 2, No. 1) separating Nitex nylon screen cloth filters of various mesh sizes (No. 2) and a metal base section (No. 3) provided with a 4-inch gate-valve-controlled outflow (not shown) and hose (No. 4) leading to the ship's scuppers. A smaller valved opening, also in the base of the unit, is used to sample the water passing the finest mesh cloth.

The filter cloths are mounted on $\frac{1}{16}$-inch flexible acrylic rings. Circular gaskets of $\frac{1}{8}$-inch neoprene are placed on either side of the rings and the filter assembly is fastened with "past-center" clamps between the lips of two of the plastic units, thus forming a water-tight sandwich. A Worthington Gamon Corp. Watch Dog bronze disc 1.5-inch water meter with enclosed gear train (Fig. 2, No. 5) provides a continuous measure of the volume of water passing into the collecting system. The connections between the collecting unit and hose are of polyvinylchloride pipe and flexible automobile radiator hose held in position with hose clamps. A by-pass (Fig. 2, No. 6) before the water meter allows for continuous pumping without passing the water through the filter cloths. Under normal operating conditions the pumped water enters the plankton collecting unit from the top and is allowed to flow by gravity through the series of cloths, which are arranged in decreasing mesh size from the top downwards. The cloths provide barriers which slow the water but still permit effectual flow through the filters, thus providing proper sorting of the organisms into size groups. A small air hole drilled in the upper part of each circular plastic unit allows for equalizing pressure throughout the system.

The cloth filters can be removed readily and placed in large volume receptacles where the organisms may be washed off using a high pressure saltwater stream directed on the face of the cloth at an oblique angle or by

"back washing." A volume of less than 1 liter is generally adequate to clear the filters of organisms.

Comparative pump and net samples. — To evaluate its sampling efficiency, the pump system was compared with conical net tows in three sets of samples from the inshore waters of the Pacific Ocean off Del Mar, California, and the Gulf of Santa Catalina. The sampling program was:

Set	Date	Position	Water volume filtered (m^3)	
			Pump	Net
I	March 11	32°59.5′N 117°19.1′W	9.78	19.63
II	March 11	32°59.0′N 117°23.8′W	7.34	19.63
III	March 14	32°32.6′N 117°33.0′W	14.34	19.63

Vertical pump samplings were made from 100 m to the surface, the pump being raised in steps of 1 to 2 m/min. The filter cloths used in the plankton collecting unit were of 103-μ, 202-μ, and 363-μ mesh. These are comparable to mesh sizes frequently used in net samplers. Two half-meter diameter nets, mounted adjacent to each other and having 103-μ and 363-μ mesh Nitex respectively, were used in vertical tows from 100 m to the surface immediately after the pump sampling. The nets were raised at a rate of 10–14 m/min. The volume of water filtered by the nets was calculated from the area of the mouth and the length of wire out, thus assuming complete filtration. Flow meter readings were not used since the rate of ascent was close to the probable friction point of the meter. No evidence of serious net clogging was indicated when the nets reached the surface. In an effort to pump a volume of water similar to the volume filtered by the nets, the time required to obtain the pump samples was generally in excess of 1 hr. During this period the boat was allowed to drift. However, conditions during the operation were ideal with smooth seas and little surface current.

The 103-μ mesh cloth was the lower limit used for the comparison since a net of the fine mesh material that we use routinely in the plankton collecting unit (i.e. 35-μ mesh) is subject to considerable and unpredictable clogging and it would have been difficult to obtain even a semi-quantitative sample. Also, it is suggested that results from a comparison at the 103-μ level would apply generally to the smaller animals since these are probably less able to avoid capture than the larger forms.

The plankton samples were preserved in approximately 5% neutral formalin. Identification and enumeration of all groups of zooplankton and representative major phytoplankton were made. The 103-μ cloth and net samples were counted using an inverted microscope at a magnification of 63×.

FIG. 2. The deck-mounted plankton collecting unit. 1, circular acrylic plastic unit; 2, nylon filter cloth; 3, metal base section; 4, hose from 4" outflow valve to ship's scuppers; 5, water meter; and 6, by-pass valve.

The procedure was based on Utermöhl (1931). Three sedimented 5- or 10-ml aliquots of the preserved materials were counted in each sample. This represented approximately 2–4% of the total sample. The 202-μ cloth and 363-μ cloth and net organisms were counted in a Bogorov counting dish (grooved dish in which the entire width of the channel can be examined in one field) at a magnification of 15×. Either $\frac{1}{4}$ or $\frac{1}{8}$ of the total sample, split with a Folsom plankton splitter, was counted (McEwen et al., 1954).

In Fig. 3 comparisons between pumped and netted samples of (1) the numbers of organisms retained by 103-μ mesh and of (2) those retained by 103-μ mesh but passing 363-μ mesh are made for the total zooplankton, total protozoa, total metazoa, and all zooplankton and phytoplankton groups which occurred in numbers greater than 300/m^3 in any sample. In the first, the

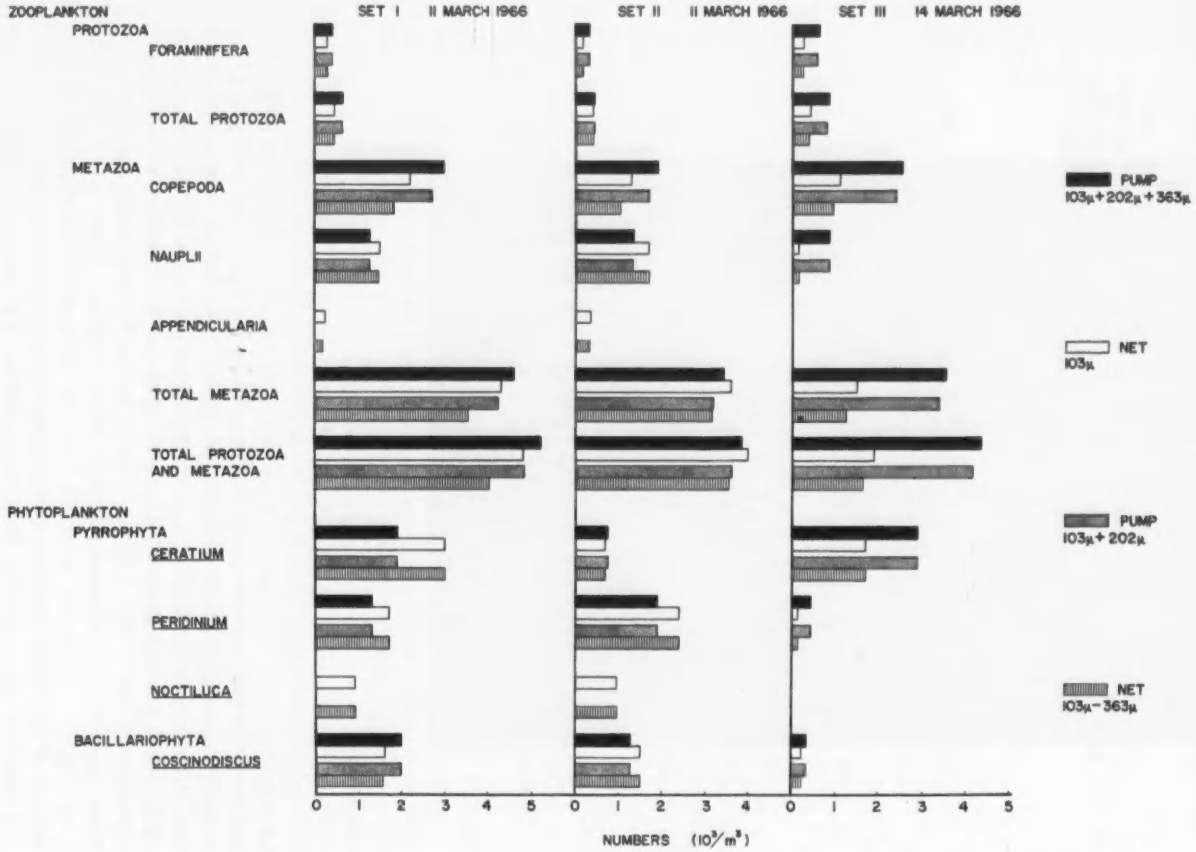

FIG. 3. Comparisons between pump and net of the numbers of organisms retained by 103-μ mesh (pump, 103 μ + 202 μ + 363 μ; net, 103 μ) and of those retained by 103-μ mesh but passing 363-μ mesh (pump, 103 μ + 202 μ; net, 103μ–363μ).

total pump samples, i.e. catches on the 103-μ + 202-μ + 363-μ mesh cloths, are compared directly with the 103-μ net catch. In the second, the data for the pump samples were determined by summing the numbers of organisms retained on the 103-μ and 202-μ filter cloths; for the net samples, by subtracting the numbers of organisms obtained in the 363-μ net from those obtained in the 103-μ net catch. If we consider only the groups of organisms which have some protective covering, in general there was less than a two-fold difference between the pump and net samples of each set collected on March 11. Appendicularia (*Oikopleura* sp.) were found in relatively large numbers in the net samples but were absent or very few in the pump catches. This form, as well as other soft-bodied organisms, was probably damaged beyond recognition in the pumped samples. The bodies of chaetognaths were likewise severely damaged by the pumping but their jaws remained intact and could be used for the group identification. The fragile dinoflagellate *Noctiluca miliaris* Suriray was found in large numbers (greater than 900/m^3) in both 103-μ mesh net tows of March 11, but not in the comparative pump samples.

The differences in the counts of the catches by the two methods of sampling were not in constant direction. For example, whereas copepods were taken in larger number by the pump, more nauplii were counted in the net samples. Dinoflagellates of the genus *Ceratium* were more abundant in the net sample from Set I while the pump sample held slightly more in Set II. The opposite was seen in the sample distribution of the diatom genus *Coscinodiscus*.

Set III of comparative samples, taken on March 14, showed a generally larger and more regular difference between the catch results of pump and net (Fig. 3). The pump, with the exception of a few groups of soft-bodied animals, consistently showed larger catches than the net. The larger differences in Set III may be the result of a high degree of patchiness of organisms and/or drift of the boat during the period of pump and net sampling at this location.

Although the assumption that the 103-μ mesh net was filtering at 100% efficiency is probably not valid, a filtration efficiency of 50–60% would still result in numbers for abundant species which would not differ by more than a factor of two from those for the pump samples in Sets I and II. The relatively slow rate of ascent of the nets may have allowed the more motile organisms to avoid the net, but this would not explain the similar differences observed in the several groups of phytoplankton.

The spatial patchiness of planktonic organisms was probably an important factor in the difference in abundance indicated by the pump and net samples. However, another contributory source towards these differences may be variable losses of the organisms through the mesh apertures of the filter cloths and nets under the two methods of sampling. The angle at which the organisms approach the opening and the way in which the aperture is held open may affect the organism's passage. In addition, the absence of bridles or other obstructions in front of the pump intake, in contrast to the usual towed net arrangement, may be an advantage in sampling certain organisms.

FIG. 3. Comparisons between pump and net of the numbers of organisms retained by 103-μ mesh (pump, 103 μ + 202 μ + 363 μ; net, 103 μ) and of those retained by 103-μ mesh but passing 363-μ mesh (pump, 103 μ + 202 μ; net, 103μ–363μ).

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Comparative Effects of a Lampricide and of Anoxia on the Sea Lamprey

The selective toxicity of the chemical 3-trifluormethyl-4-nitrophenol (TFM) for lampreys was postulated to be due to its interaction with oxygen transport, which is more sensitive in the lamprey than in other fish (Howell, personal communication). Oxygen transport in the lamprey might be affected by either the production of methemoglobin or by high acidity in the blood, both of which cause anoxia. These effects were postulated because (1) Christie and Battle (1963) found that upon exposure to lethal doses of TFM, the lamprey showed vasodilation of the gill filaments and liver, and edema of the connective tissues between the respiratory epithelium and the vascular endothelium; (2) nitro-aromatic compounds oxidize hemoglobin to methemoglobin; (3) TFM is a fairly strong acid with a pKa of 6.07 ± 0.03 (Smith et al., 1960) as compared to phenol, pKa 9.98, and acetic acid, pKa 4.76; and (4) in the hemoglobin studies conducted by Wald and Riggs (1951), Allison et al. (1960), Briehl (1963), Rumen and Love (1963), and Antonini et al. (1964) it was found that lamprey hemoglobin had some unique properties, including a large Bohr effect.

The present study using electrocardiograms was undertaken to determine whether or not the effect of TFM and the effect of anoxia on the electrophysiological events of the sea lamprey heart were of the same nature. In the last 20 years there have been only two electrocardiographic (ECG) studies of the lamprey (Oets, 1950; Ten Cate and Punt 1960) and both were with the species *Lampetra fluviatilis*. Oets commented on the ability to pick up the ECG from electrodes placed on the skin, but Ten Cate and Punt did not obtain satisfactory results from electrodes on the skin, and had better ECG recordings with the electrodes placed on the pericardium. In the present study electrocardiograms were taken of postlarval lampreys while their gills were being perfused with aerated water containing TFM and while they were given unaerated water to produce anoxia. The results indicate that TFM produces changes in the electrophysiological events of the lamprey heart different from those observed following anoxia.

To facilitate handling, the lampreys were anesthetized in M.S. 222 at a concentration of 0.065 g/liter of spring water as suggested by Thorson (1959). A lamprey was placed in 500 ml of the anesthetic which was continually aerated. All muscular movement, except in respiration, ceased in approximately 5 min. The lamprey was then placed on a board. Aerated fresh spring water or TFM solution was run from a separatory funnel through a "feeding" tube. This tube was placed in the lamprey's mouth, and an opaque rubber tube was placed over the lamprey's head and tied to the board so that the animal had its ventral side up. A tube was then placed over the tail and tied to the board leaving only the gill region and the heart area posterior to the gills

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uncovered. The water flowing into the perfusion tube was regulated with the stopcock until a steady respiratory movement was observed. The electrodes, hypodermic needles, were then pressed into the body cavity near the pericardium, the positive electrode placed on the right or ventricular side, and the negative on the left or atrial side. A thermistor was placed in the water flowing from the gill slits. Water temperature, ECG, and respiratory movements were recorded simultaneously by a Physiograph "Six" (E. & M. Instruments). Respiratory traces were taken to ascertain the instrument's electrical separation of the heart potentials from the respiratory potentials.

The recordings indicated that (1) the best ECG was obtained with the electrodes adjacent to the pericardium, and that only insignificant results could be obtained from the skin; (2) the recorder's separation of the respiratory potentials from the heart potentials was efficient even when only one set of electrodes was used for recording both potentials; (3) the changes in water temperature during all the studies were too small to be considered as significant factors in altering the ECG; (4) the nature of the atrial and ventricular potentials of the heart was similar to that recorded by Ten Cate and Punt (1960); and (5) the resultant potentials of the lamprey heart follow a vector away from the sinus venosus, and toward the ventricle.

For a control study an anesthetized lamprey was placed on the apparatus and continuously perfused with aerated spring water. The recordings for this experiment were similar for over 200 min. Therefore, the apparatus was recognized as workable, and the effects on the lamprey as minimal and insignificant for at least this amount of time.

Before treatment with TFM, two lampreys were perfused with aerated spring water for 122 and 126 min, respectively, to insure that the anesthetic had worn off. Aerated spring water containing 3 ppm lampricide, a concentration which kills 100% of the larval, post-larval, and adult lampreys (Applegate et al., 1961), was then allowed to flow through the perfusion tube. Recordings were taken until the lampreys died at approximately 60 and 90 min, respectively, after the initiation of the perfusing with TFM.

Anoxia was produced in lampreys by stopping the aeration of the water. To test whether or not anoxia produced a similar ECG as TFM, a lamprey was perfused with aerated spring water for 120 min, and then perfused with unaerated spring water. Changes were seen within the first 30 min. The lamprey did not die after 111 min of unaerated water perfusion, but the recordings showed significant changes from that of the TFM and control studies.

Electrocardiograph studies directly indicate heart potential direction, duration, and deviations, and only indirectly heart contraction and its forces and work performance — all considerations beyond this being purely assumptions (Schaefer and Haas, 1962). These results (Fig. 1) indicate that there are three significant differences between the ECG recording produced during the unaerated water study and those produced in the TFM studies. These differences are (A) a continual increase in the height and area of the P wave in the unaerated water study, but a steady height with TFM; (B) a

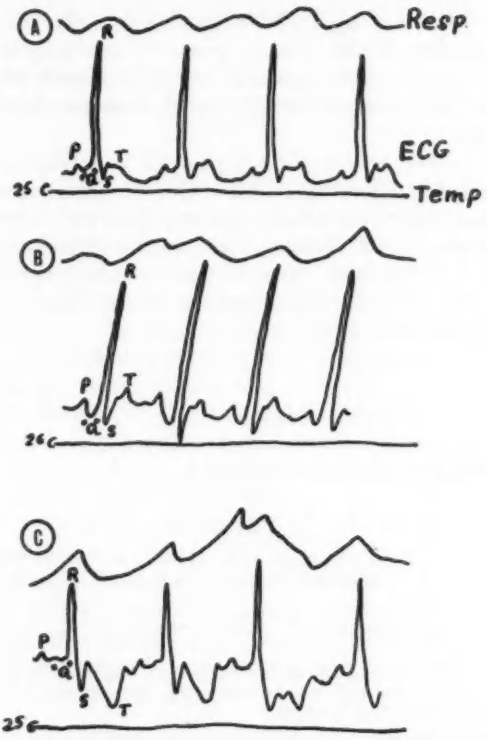

FIG. 1. Respiratory potentials (Resp), electrocardiograms (ECG), and thermistor recordings (Temp) of: (A) the control study after 180 min of perfusion with aerated spring water; (B) the anoxia study after 40 min of perfusion with unaerated spring water; (C) one of the TFM studies after 40 min of perfusion with 3 ppm TFM (0.1 mv = 1.6 cm, 1 C = 0.3 cm, chart speed = 5 cm/sec).

continual increase in the height and area of the "Q" RS wave (the Q is missing in the lamprey ECG because there is no ventricular septum) with unaerated water, but a decrease with the TFM studies; and (C) an increase in the height and area of the T wave with the unaerated water, but an inversion of the T with the TFM studies. This increased potential of the lamprey heart during the unaerated water study indicates stronger heart muscle contraction in an effort to increase cardiac output and the rate of blood passing through the gills, thus increasing the oxygen in the blood. The inversion of the T wave incurred with the use of TFM is indicative of drug action (Schaefer and Haas, 1962). All the lampreys killed with TFM were found to have red blood indicating the absence of methemoglobin. Vasodilation in the gills and liver was

consistent with TFM having a $-NO_2$ functional group and that nitrites are vasodilators (DiPalma, 1964). The present electrocardiographic study, therefore, shows that the effects of anoxia and TFM on the electrophysiological events of the lamprey heart are not the same, whatever those effects, direct or indirect, may be.

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Effect of Ovine Prolactin on Tissue Electrolyte Composition of Hypophysectomized Freshwater Eels (*Anguilla rostrata*)

A number of species of Cyprinodont fishes are unable to live in freshwater after hypophysectomy. These include *Fundulus heteroclitus* (Pickford and Phillips, 1959), *Poecilia latipinna* (Ball et al., 1963), and *Xiphophorus maculatus* and *X. hellerii* (Schreibman and Kallman, 1963). Injections of mammalian prolactin after hypophysectomy will promote freshwater survival in at least one of these species, *F. heteroclitus* (Pickford and Phillips, 1959; Pickford et al., 1965). In intact *F. heteroclitus* (Burden, 1956; Ball and Pickford, 1964) and *Poecilia* (Ball and Olivereau, 1964) enlargement of the erythrosinophilic cells of the rostral pars distalis occurs during adaptation to freshwater. These cells produce a teleostean prolactin (Chadwick, 1966; Emmart et al., 1966). There is increasing evidence that this hormone is important for osmoregulation in some teleost fishes although little is known about its site of action.

In contrast, hypophysectomized yellow eels (*Anguilla rostrata*) are able to survive in freshwater (Fontaine et al., 1949; D. G. Butler, unpublished observations) although the operation is followed by a gradual depletion of tissue electrolytes (see Table I). The present work attempts to show whether or not ovine prolactin will maintain or reestablish normal serum and muscle electrolyte concentrations in hypophysectomized eels. Mechanisms for osmoregulation are not considered here.

TABLE I. Tissue electrolyte composition of hypophysectomized immature freshwater eels after treatment with ovine prolactin.

		Serum (*meq/liter*)			Muscle (*meq/kg muscle water*)			
Group	*n*	Na	K	Cl	Na	K	Cl	% tissue water
Intact	10	153.6 ±0.12	4.74 ±0.49	113.7 ±1.7	35.2 ±2.2	154.0 ±10.4	17.21 ±1.19	60.6 ±0.93
HypoX 3 weeks—controls	10	143.4** ±1.0	6.00 ±0.61	103.3** ±1.6	32.3 ±0.94	140.4 ±3.1	14.00** ±0.60	61.7 ±0.85
HypoX 3 weeks—prolactin	10	137.0 ±0.99	6.10 ±0.46	98.4 ±2.9	31.5 ±1.6	138.7 ±2.8	15.66 ±1.15	63.4 ±1.1

** $P<0.01$ when group II is compared with group I. There are no significant differences between values in group III and group II. All values are means ± SE.

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Immature female yellow eels (*Anguilla rostrata*) weighing approximately 1000 g each were collected from the Bay of Quinte, Lake Ontario in June 1966. Eels were transported to aquaria and held in running dechlorinated tap water (10 C) without feeding, for at least 2 weeks before they were used for experiments. A number of eels were anaesthetized with an aqueous (3 g per liter) solution of M.S. 222 (ethyl *m*-aminobenzoate methanesulphate, Kent Chemicals, Vancouver) and hypophysectomized (Butler, 1966).

Three groups of eels were used for experiments. Group I consisted of intact eels; groups II and III of hypophysectomized eels. During the last 10 days of a 3-week period, eels in group II were given daily intramuscular injections of 0.1 ml of distilled water. Animals in group III (Table I) were given corresponding injections of distilled water each containing one IU ovine prolactin (NIH-PS7, mean potency 24.3 IU/mg) per kg body weight.

It seemed important to test the effect of smaller and possibly nearer physiological doses of prolactin than formerly used to promote survival of hypophysectomized *Fundulus heteroclitus* (Pickford et al., 1965). Table I shows that, under given conditions, ovine prolactin will not prevent tissue electrolyte depletion from hypophysectomized North American eels. Larger doses, approximately 480 IU ovine prolactin (NIH-PS6) per kg body weight per day, administered from the time of hypophysectomy (Olivereau and Chartier-Baraduc, 1966) delayed but did not prevent serum sodium and potassium depletion from male silver European eels (*Anguilla anguilla* L.). In these eels the delay in serum sodium and potassium depletion after treatment of hypophysectomized eels with prolactin could have been due to a shift of these ions

TABLE II. Intracellular sodium and potassium concentrations and distribution of muscle water.

		Fluid (*g/kg muscle water*)		Intracellular ions (*meq/liter*)	
Group	n	Extracellular	Intracellular	Na	K
Intact	10	84.8 ±2.6	915.2 ±1.5	15.2 ±1.7	161.9 ±7.4
HypoX 3 weeks— controls	10	83.2 ±2.4	916.8 ±3.2	14.3 ±0.69	160.7 ±3.6
HypoX 3 weeks— prolactin	10	91.1 ±4.3	909.9 ±5.2	12.2 ±1.3	161.8 ±4.3

All values are means ±SE. Ion concentrations in extracellular water (chloride space) were calculated assuming that sodium, potassium, and chloride concentrations in extracellular fluid were the same as those in serum.

from cells to extracellular fluid. Therefore, in the present experiments extracellular (chloride) space was estimated for epaxial muscle excised dorsolaterally at the level of the cloaca. Muscle electrolyte concentrations and per cent water were measured using standard methods (see Butler and Langford, 1967). Chloride space (grams/kilogram muscle water) and intracellular sodium and potassium concentrations were calculated using formulae given by Gordon (1959). Data in Table II show that extracellular space is small and remarkably constant for eels in all experimental groups. Similar extracellular fluid volumes have been recorded for other freshwater teleosts (Fontaine, 1951; Gordon, 1959; Houston, 1959).

Intracellular potassium concentrations did not change in hypophysectomized eels compared with intact controls or hypophysectomized eels injected with prolactin compared with hypophysectomized controls (Table II). Intracellular sodium may have decreased in hypophysectomized eels with and without prolactin although the changes were not statistically significant. Serum sodium depletion from eels would then represent a loss of sodium from intracellular as well as extracellular fluids; chloride loss is apparently extracellular.

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Effect of Aliasing on Studies of Long-Term Oceanic Variability off Canada's Coasts

In recent years three sets of oceanographic measurements have been made on a regular basis off Canada's coasts. These are the series of observations at Ocean Station P in the Pacific which commenced in 1952; a line of stations between Ocean Station P and Swiftsure Bank occupied since 1956; and a line of stations running southeast from Halifax over the continental shelf into deep water (the Halifax section) occupied since 1950. If these series are to be used to support studies of long-period phenomena, it is pertinent to enquire whether the rate of sampling is high enough to prevent "aliasing."

Geophysical phenomena are often sampled at equal intervals of time (Δt). If the phenomenon does not oscillate at frequencies greater than the folding frequency, $f_N = 1/(2\Delta t)$, a power spectrum analysis of the data determines the power in bands centered about frequencies less than f_N. The width of the frequency bands (resolution) and the reliability of the estimates of the power in them (stability) depend on the length of the data record. If the phenomenon, however, has frequencies greater than f_N, power spectrum analysis of the data results in the power of frequencies greater than f_N appearing in frequency bands centered about frequencies less than f_N and the spectrum is said to be aliased. More specifically, the power spectrum analysis results in the frequency band centered about frequency f below f_N not only containing the power naturally occurring in the band but also containing the power from all the bands above f_N centered about the frequencies $(2k\,f_N \pm f)$, k integer. The spectrum above f_N is said to be folded back on the interval 0 to f_N. This is illustrated in Fig. 1. The amount of aliasing is independent of the length of the record. For a detailed account see Blackman and Tukey (1958).

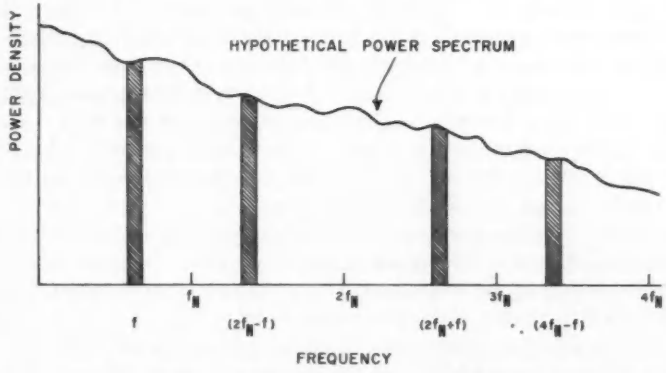

FIG. 1. A hypothetical power spectrum. Power in the bands centered at frequencies $2kf_N \pm f$ appears in the band centered at f if the sampling period is $\Delta t = \frac{1}{2}f_N$.

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From the above it is apparent a power spectrum is of little value unless aliasing by high frequencies is sufficiently small. The amount of aliasing depends on the sampling rate. The higher this is, the higher is f_N and the less the power folded back into the spectrum below f_N. To estimate whether aliasing is serious for a given sampling rate, one must compare the natural power density in the band of interest with the natural power density in the high frequency bands which are folded onto the band of interest by power spectrum analysis. Although such a comparison cannot usually be made because knowledge of the high-frequency variations is often lacking in the open ocean, station P provides one instance where a crude estimate of aliasing can be made.

At Station P twice-weekly measurements of temperature and salinity are made to depths of 400 m. These measurements are made every alternate period of 6 weeks since only one weathership is equipped to make them. From the data, Tabata (1965) has calculated the six-weekly average temperatures on several σ_t surfaces. When plotted as a function of time from 1957 to 1962, these average temperatures seem to show variations with frequencies of about $\frac{1}{2}$ cpy (cycle per year) and composite amplitude of about $\frac{1}{2}$ degree C. The total power of such variations is approximately equal to $\frac{1}{2}$ (amplitude)^2, or $\frac{1}{8}$ degree C^2. If this power is spread over a band $\frac{1}{2}$ cpy wide centered at $\frac{1}{2}$ cpy, the power density is about $\frac{1}{4}$ degree C^2/cpy. If the power is concentrated at some particular frequency in the band, the power density near the frequency may be somewhat greater. It should be pointed out, however, that a record of at least 20 years is needed to obtain the resolution necessary to reliably separate the power in the band from the power at frequencies greater than $\frac{3}{4}$ cpy and less than $\frac{1}{4}$ cpy (periods less than $1\frac{1}{3}$ years and greater than 4 years). The distribution of the power in the band can only be obtained from much longer records.

Let us now consider whether the power density due to aliasing is significant in the frequency band of interest. From the plot of one 6-week period of the twice-weekly values of temperature (T) on σ_t (Tabata, 1965, fig. 16), it is apparent that fluctuations of T with composite amplitude of about $\frac{1}{2}$ degree C and with frequencies greater than 2 cycles/week or 104 cpy are present. In the same way as previously we estimate the total power of these variations as $\frac{1}{8}$ degree C^2. Since changes of T on σ_t are due to advective processes, it also seems likely that there is little energy at frequencies much greater than 1 cycle/day or 365 cpy. Thus, assuming the power to be equally spread in the band, we estimate the power density in the band from 104 cpy to 365 cpy to be $[8(365-104)]^{-1}$ degree C^2/cpy $\approx$ 0.0005 degree C^2/cpy.

If both weatherships were used and the measurements were still made twice weekly, we would have a folding frequency of 52 cpy. Then the spectra would be aliased by power in the band from 52 to 365 cpy, which folds 6 times onto the band from 0 to 52 cpy. Using the power density calculated above, this gives a power density at low frequency due to aliasing of about 0.003 degree C^2/cpy. Since this is about one-eightieth of the estimated power density of the variations with frequencies about $\frac{1}{2}$ cpy whose nature we wish to investigate, sampling regularly at twice-weekly intervals is adequate.

Let us now consider the present situation where measurements are only made every second 6-week period. These data may be handled by averaging over the 6-week period and then performing a power spectrum analysis on the averaged values.[1] The effect of the averaging process is to apply to the spectrum (including the aliases indicated above) a filter which only allows significant transmission at frequencies lower than 1 cycle/6 weeks, i.e. 8.7 cpy. Because of the gaps in the data, however, after averaging there only is one data point every 12 weeks and the new folding frequency is thus 2.2 cpy. This means that a power spectrum analysis results in the power transmitted in the band from 2.2 to 8.7 cpy being folded back 3 times onto the band from 0 to 2.2 cpy. If the power density in the band from 2.2 to 8.7 cpy was equal to the value of 0.003 degree C^2/cpy due to high frequency aliasing, the power density below 2.2 cpy due to aliasing would only be about .009 degree C^2/cpy. This is still much less than the estimated power density of the low frequencies we wish to investigate. The power density due to natural variations in the band from 2.1 to 8.7 cpy, however, is not expected to be negligible. In fact, since geophysical spectra tend to rise more or less continuously towards lower frequencies, the power density in the band from 2.2 to 8.7 cpy may be a significant portion of the power density in the band of interest. Thus, aliasing due to the power in the natural frequencies in this band may be considerable and analysis of the averaged data is not to be recommended.

The above estimates are, of course, crude but they do show the definite advantage of instrumenting both weather ships. As Fofonoff (unpublished data) and Tabata (1965) have already pointed out, this will also give a more complete coverage of the spectrum. The above calculation indicates also that the sampling rate that can reasonably be expected for the section to station P from Swiftsure Bank (8–12 times per year) is probably not high enough.

We have no suitable data to make even a crude numerical estimate for the Halifax section. This section is normally run four times each year. In 1962 it was run 12 times. A plot of temperature on the surface $\sigma_t = 27.3$ at a station off the continental shelf is shown in Fig. 2 for the years 1961 through 1963. This surface lies at depths around 250 m, below the seasonal thermocline. Temperatures and salinities are determined by advection of warm (8 C) water derived from the Gulf Stream to the south and cool (5 C) water derived from the Labrador Current to the north. If this advective process has any long-term cycle it looks as though the resulting change of T on σ_t will be less than 2 degree C. As can be seen from Fig. 2, changes of this magnitude can occur in a month. Hence in planning a time series to ascertain if long-term changes exist, we must assume that a considerable amount of power is present at high frequencies and sample at a high rate. Certainly four samples per year is far from adequate.

It should be noted that the estimate of aliasing given above is based on temperature variations on σ_t surfaces and that for this case we were able to

[1]Although this is the usual way to handle such data, it may be that special techniques could be developed to give better results.

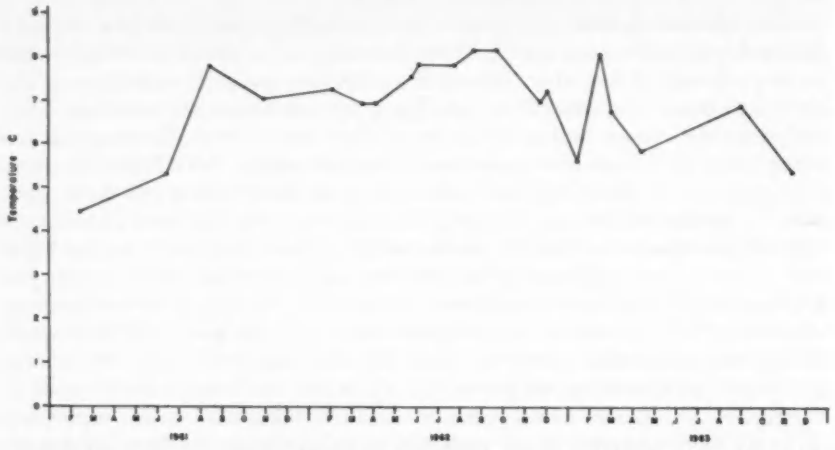

FIG. 2. Temperature on the surface σ_t = 27.3 from 1961 to 1963, for a station just off the continental shelf southeast of Halifax.

assume that the power spectra fall off quickly for frequencies greater than 365 cpy. Other variations, for example, of temperature at constant depth, may have considerable energy at frequencies greater than 365 cpy. In this event high-frequency aliasing may be more serious than estimated above. This will be especially the case if the high-frequency spectrum has sharp peaks at certain frequencies such as the Väisälä frequency. It is apparent that the sampling rate must be carefully chosen for each particular problem if aliasing is to be avoided.

We conclude by remarking that the time series at Station P should be useful in studying certain long-term changes if both ships are instrumented. On the other hand the sampling rate that can reasonably be maintained by ships along the section to station P is so low that for the above purpose moored buoys should be used. This is being done with the Halifax section commencing in January 1967. In addition to enabling the sampling rate to be increased, moored buoys have the advantage that instruments are left in situ for the length of the mooring. This means that aliasing may be completely avoided for certain measurements by the use of instruments that have time constants commensurate with the sampling rate. For example, for temperature measurements one can use a thermometer enclosed in a case of material of low heat conductivity which can be made thick enough so that the thermometer only senses temperature variations with periods which are longer than the sampling interval.

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Germination of Spores of *Clostridium botulinum* Type E in Fish and Shellfish Extracts

In a recent publication (Strasdine, 1967) we reported on the ability of spores of *Clostridium botulinum* Type E to undergo a rapid and complete germination in trypticase–peptone media under suitably defined conditions of *p*H, temperature, and nutrient concentration. We have since extended these findings to include various fish and shellfish extracts with the purpose of obtaining a better understanding of the mechanisms of spore germination in media of marine origin. In the following communication it will be seen that several tissue extracts are equal to or better than the trypticase–peptone media in promoting rapid germination (30–60 min) of Type E spores.

Water-soluble extracts were prepared from fresh fillets of salmon (*Oncorhynchus kisutch*), sole (*Parophrys vetulus*), lingcod (*Ophioden elongatus*), and Pacific herring (*Clupea pallasii*) by homogenizing each tissue with an equal part (w/v) of physiological saline. Extracts of fresh Pacific shimp, crab, and oyster were similarily prepared. Homogenates were centrifuged to remove insoluble tissue, boiled 10 min, and centrifuged; the clear supernatants were then collected. Protein that was precipitated by repeated freezing and thawing of these extracts could be removed without loss of germinating activity. Germination rate measurements, determination of rate constants, and the plotting functions employed have been described (Strasdine, 1967). Amino acid nitrogen was determined by the method of Cocking and Yemm (1954). Optical density changes were made directly in 14 × 170-mm Thunberg tubes. Each tube contained 1.0 ml of tissue extract; glucose, 0.02 M; 3×10^8 spores; and Tris buffer, 0.1 M *p*H 7.0. Distilled water was added to a final volume of 4.0 ml.

The ability of the various tissue extracts to support spore germination is shown in Fig. 1. Spores germinated rapidly in the presence of the cod extract; salmon, sole, and to a lesser degree herring, also demonstrated good activity. A comparison of the germination rate constants (k_o), on the basis of activity per gram of tissue, shows cod to be more than twice as active as any other tissue examined in promoting rapid spore germination (Table I). Extracts of shrimp, oyster, and crab were relatively inactive in allowing rapid germination.

As with the trypticase–peptone medium, in each case the tissue fraction allowing rapid germination was dialyzable, was destroyed on ashing, and was shown to contain a wide spectrum of free amino acids and peptides. A complete quantitative amino acid analysis of the active fractions from each tissue failed to show any correlation between the concentration of any single or group of amino acids and the germinating activity of that tissue. At present the quantitative relation of those examined that appears most consistent in determining the ability of a given tissue to support rapid germination is the ratio of peptide

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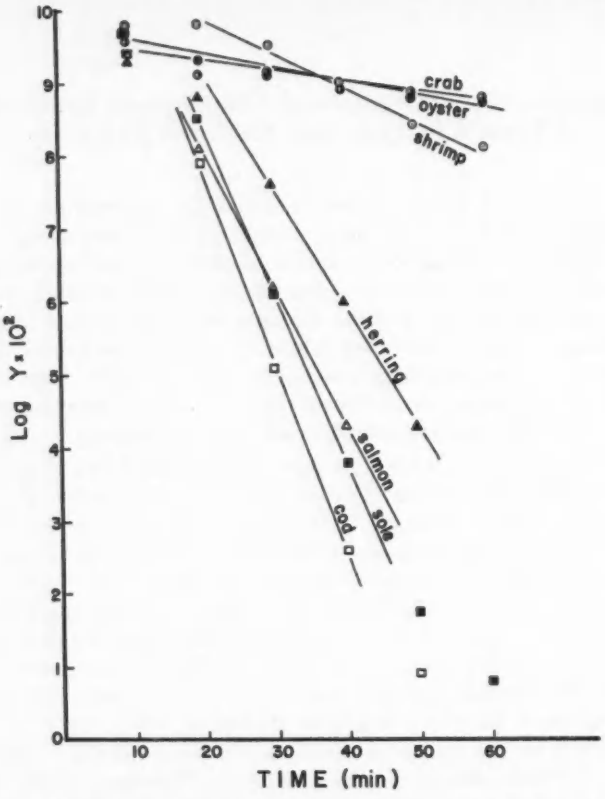

FIG. 1. Germination of *C. botulinum* Type E spores in fish and shellfish extracts. $Y = OD - OD_f / OD_i - OD_f$ where OD is the optical density at time t; OD_i and OD_f are the initial and final optical densities respectively.

TABLE I. Comparison of amino acid–nitrogen concentration and germination rate constants for tissue extracts.

Extract	k_o/g[a] tissue	mg amino acid nitrogen per g of tissue		$\frac{a}{b}$
		(*a*) peptide	(*b*) free	
Cod	2.81	.584	.086	6.8
Sole	1.20	.296	.072	4.1
Salmon	0.82	.235	.060	3.9
Herring	0.64	.353	.097	3.6
Shrimp	0.32	.723	.457	1.6
Oyster	0.28	1.844	.966	1.9
Crab	0.20	1.068	.732	1.5

[a] k_o (the germination rate constant) was obtained from the slopes of the curves in Fig. 1.

concentration to free amino acid concentration (Table I and Fig. 2). Germination rate constants for the trypticase–peptone media are also consistent with this observation.

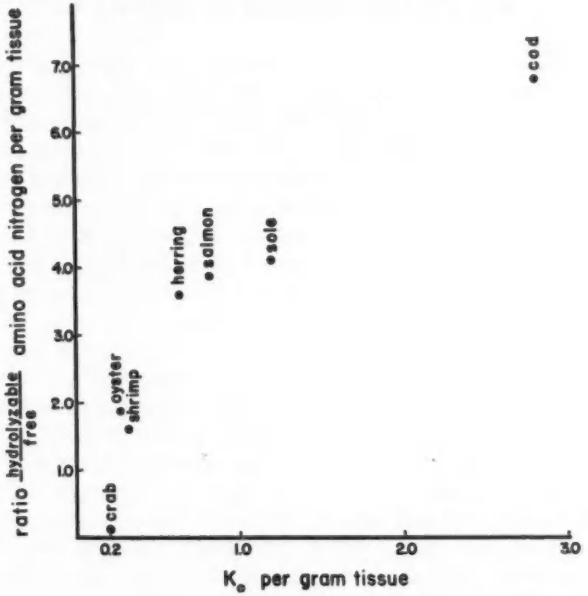

FIG. 2. Effect on germination rate of the ratio of concentration of free amino acid to dialyzable peptide in tissue extracts.

Extracts from frozen or aged fillets contain higher concentrations of either or both free amino acids and dialyzable peptides. If the ratio of the concentrations remains constant, there is little if any change in the germination rate constant. Frequently, however, the free amino acid concentration is increased to a greater extent than is the peptide concentration and in these extracts the germination rate constants decrease proportionately.

It should be noted that in these experiments, we have not considered the ability of the tissue extracts to support spore outgrowth, vegetative cell growth, or toxin formation.

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Nucleotide Degradation, Monitored by Thin-Layer Chromatography, and Associated Postmortem Changes in Relaxed Cod Muscle

Although the rate and extent of postmortem nucleotide degradation, leading ultimately to the accumulation of hypoxanthine, has been established for several species of trawled (exhausted) fish during iced storage (see Jones, 1965), no comparable studies have been made of fish muscle completely relaxed at death. Such relaxed muscle, taken from fish which have undergone virtually no antemortem muscular activity or stress, contains high initial levels of CP[1] and ATP, and little or no lactate (Fraser et al., 1966a). For such an investigation, we sought a rapid procedure for following nucleotide breakdown in greater detail than that afforded either by the nonspecific ultraviolet absorption examination of neutralized perchloric acid extracts or measurement of acid-labile phosphorus. Column chromatographic procedures (Kassemsarn et al., 1963), which have been used to achieve excellent separations of the five major nucleotides present in fish muscle (ATP, ADP, AMP, IMP, DPN) and of the degradation products inosine and hypoxanthine, are unwieldy and time-consuming. Thin-layer chromatography appeared to offer the rapid resolution required. The technique has been applied successfully to the separation of complex standard mixtures of nucleotides, and also to mixtures of nucleosides and to mixtures of bases (Randerath, 1966). There have been few attempts, however, to separate standard mixtures containing a combination of these three groups of purine compounds (e.g. Pataki and Kunz, 1966) or the purines in biological systems such as neutralized perchloric acid extracts of muscle. Using anion-exchange chromatography on 0.5-mm layers of poly(ethyleneimine)-cellulose applied to plastic sheets (Randerath and Randerath, 1966) we found that rapid and complete separation of the major nucleotides, nucleosides, and bases in such extracts could be achieved. The chromatograms were developed first in distilled water to effect resolution of Ino and Hx (nucleotides remain at the origin under these conditions), followed, after intermediate drying, by a second development in the same direction with increasing concentrations of LiCl to resolve nucleotides (see details in caption to Fig. 1). We also determined the concentrations of ATP, ADP, and IMP by cutting out the spots, eluting them, and measuring the absorbance (Fig. 2).

This thin-layer chromatographic technique proved successful in monitoring the sequence of postmortem nucleotide degradation at 0 C in fillets from cod (*Gadus morhua*) with muscle relaxed at death through gentle anaesthesia (procedure of Fraser et al., 1966a) (Fig. 1). Only 2–3 hr were required to obtain

[1]The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; IMP, inosine monophosphate; DPN, diphosphopyridine nucleotide; Ino, inosine; Hx, hypoxanthine; CP, creatine phosphate; IP, inorganic phosphate; Δ7P, acid-labile phosphorus; TN, total adenosine and inosine compounds; AN, adenosine compounds.

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clear resolution of the major nucleotides and of Ino and Hx by the systems employed. It was necessary to ensure that the *p*H's of the concentrated extracts before spotting were similar, e.g. 7.0–7.5, since mobility of the nucleotides was affected by *p*H. The detailed progression of changes, revealed by the changing intensity of the spots, is clearly evident; ATP, the predominant nucleotide at death, is dephosphorylated and deaminated to IMP within 3 days, followed by the gradual dephosphorylation of IMP in turn to Ino, which is then itself degraded to Hx, the predominant purine at 11 days. The absence of spots running in the positions of DPN and AMP indicated, by comparison with varying amounts of standards, that these nucleotides could not have been present in relaxed cod muscle in amounts greater than 0.2 μmole/g during the postmortem period. Similarly, very low levels of DPN and AMP in rested cod muscle, 0.1 and 0.7 μmole/g, respectively, were reported by Jones and Murray (1961) using column chromatography.

In relaxed cod muscle stored in ice, the high initial level of ATP, about 5 μmole/g, was maintained to 24 hr postmortem (Fig. 2*a* and Table I). Maintenance of ATP for as long as 24 hr is in contrast to the immediate and rapid disappearance of the nucleotide within 12 hr, observed in "rested" cod muscle by Jones and Murray (1961); initial IMP levels of 1 μmole/g, however, suggest that the latter fish may not have been in a truly rested condition at death. More recently, maintenance of initially high levels of ATP for 10–12 hr have been reported for unexercised trout muscle (Tomlinson et al., 1964) and for rested cod muscle (Jones et al., 1965). In the relaxed cod (present experiment) the high and constant level of ATP was undoubtedly maintained, through resynthesis, by the appreciable amounts of CP (Table I) present in the muscle initially, as in immobilized (relaxed) mammalian muscle, where ATP did not fall until the supply of CP was exhausted (Bendall, 1960). As with mammalian muscle, these initial conditions of high CP and ATP levels delayed the onset of rigor in relaxed cod to 48 hr postmortem, compared with 12 hr and 6–7 hr

TABLE I. Content (μmole/g) of certain glycolytic metabolites in relaxed cod muscle stored in ice.[a]

Time postmortem	CP	Δ7P[b]	IP	TN	AN
0 hr	7.2	16.8	32.0	8.5	6.9
5 hr	13.0	11.1	28.0	8.6	6.9
24 hr	4.8	9.2	35.0	8.6	5.8
48 hr	0	5.4	49.3	9.1	3.0
3 days		0.8	50.8	8.3	0.9
4 days			56.0	8.2	0.6
7 days			57.9	8.8	
9 days			56.9	7.3	
11 days			61.0	7.2	

[a]The fillets were stored in polythelene bags buried in ice.
[b]Excluding CP.

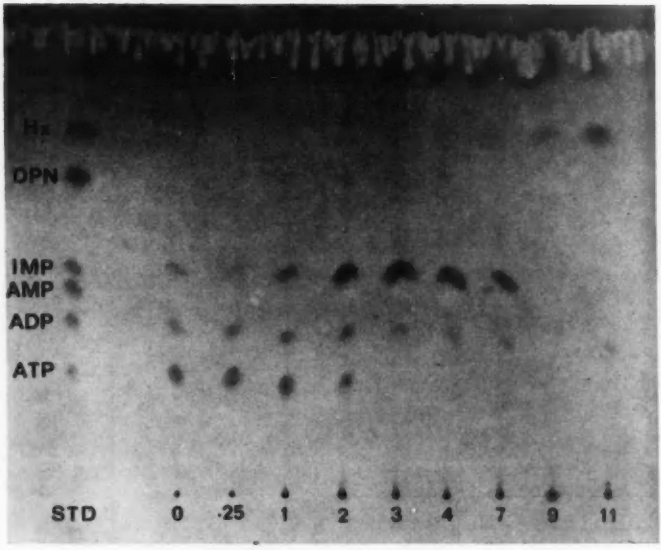

FIG. 1. Thin-layer chromatogram of the progression of nucleotide degradative changes in relaxed cod muscle stored at 0 C.

Portions of neutralized $HClO_4$ extracts (see Fig. 2) were concentrated to $\frac{1}{10}$ their volume under vacuum at 30 C and filtered to remove perchlorate. Aliquots of 0.01 ml, containing 0.03–0.05 μmoles of purine compounds, were spotted 3.0 cm from the lower edge of the PEI-cellulose coated sheets, previously washed with NaCl and H_2O to remove impurities from the cellulose (Randerath and Randerath, 1966). *First development*: distilled water to 12 cm above the starting line (60–70 min), followed by drying in a current of air at room temperature and viewing under ultraviolet light to locate Ino and Hx. *Second development*: in the same direction with 0.2 M LiCl to 1 cm, 1.0 M LiCl to 4 cm, 1.6 M LiCl to 13 cm (stepwise elution, no intermediate drying; about 75 min) (Randerath and Randerath, 1964). The chromatogram was again dried and the spots located under UV light. (The limit of detection under ultraviolet light was about 0.5 mμmole.) During the second development Hx and Ino migrate further, the latter at the front. The standard mixture contained 10 mμmoles of each compound. Photographed with ultraviolet light.

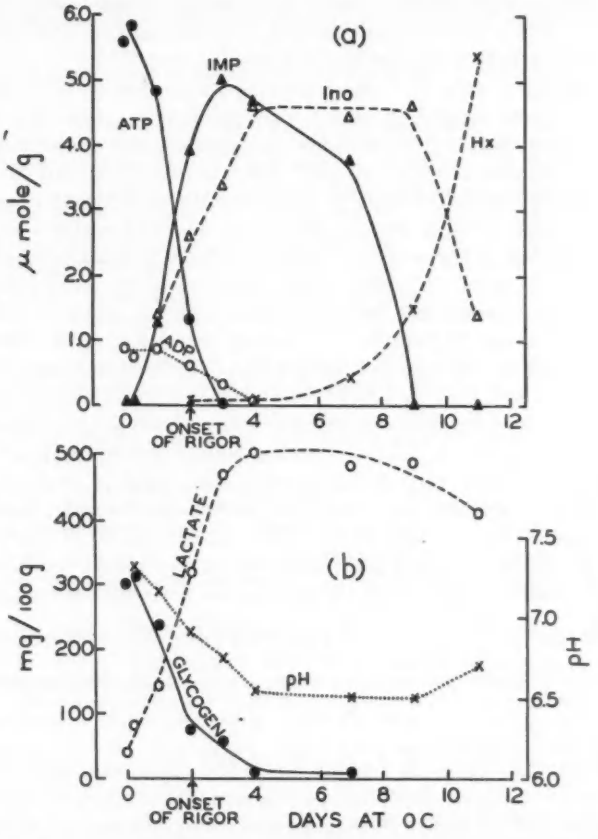

FIG. 2. (*a*) Nucleotide degradation in relaxed cod muscle stored at 0 C; ATP, ●; ADP, ○; IMP, ▲; Ino, △; Hx, ×; (*b*) the accompanying glycolytic changes.

Samples (5 g) were cut successively from the central longitudinal strip of muscle, avoiding the anterior, posterior, and ventral regions as well as the red muscle. Preparation of $HClO_4$ extracts and analyses for glycolytic metabolites (Table I), and for lactate, Ino, and Hx were carried out as described previously (Fraser et al., 1966a; Dyer et al., 1966). Glycogen was determined as the sum of that in the $HClO_4$ extract and in a KOH digest of the residue (Fraser et al., 1966b). Homogenates of 1–2 g of muscle with 10 vol of 0.005 M sodium iodoacetate were used for *p*H measurement. ATP, ADP, and IMP were determined after thin-layer chromatography by elution from their respective spots (cut out) with 2 N HCl, followed by spectrophotometric measurement of the absorbance against similarly treated blanks from adjacent areas of the sheet, using the appropriate extinction coefficients (Ratcliff and Follett, 1966). Under these conditions, recoveries for ADP and IMP standards ranged from 95 to 105%.

for cod in which ATP fell from the point of death (Fraser et al., 1961; Jones and Murray, 1961). ADP in the relaxed muscle, present at a relatively low level initially, decreased rapidly postmortem.

Concomitantly with the rapid dephosphorylation of ATP after 24 hr, levels of IMP rose rapidly to about 5 μmole/g at 3 days. The amounts of nucleotides determined in the chromatographic eluates were consistent with the apparent dephosphorylation and deamination indicated by Δ7P and ultraviolet absorption data (Table I). Subsequently, IMP was dephosphorylated rapidly, with peak levels of Ino attained at 4 days and maintained to 9 days. Degradation of Ino to Hx followed, with very rapid accumulation of the latter between 7 and 11 days, coincident with the onset of bacterial spoilage in the fillets as indicated organoleptically, and as previously observed (Kassemsarn et al., 1963). The high Hx levels were reached somewhat earlier than at the 16 days reported for trawled, gutted cod (Jones, 1965). It is seen (Fig. 2*a*) that the sum of the nucleotide catabolites is lower at 9 and 11 days; it was noted during sampling that the fillet had become soft with some apparent loss of fluid at this time, probably resulting in a loss of soluble constituents.

The accompanying glycolytic changes, completed in 4 days (Fig. 2*b*), were considerably slower than those observed in "rested" aquarium cod (approximately 30 hr) (Fraser et al., 1961). Acidification of the muscle was reflected in a fall in *p*H from 7.3 initially to an ultimate level of 6.5. Interestingly, the linear relation between *p*H fall and lactate accumulation which exists for mammalian muscle, $\frac{\Delta \text{ lactate}}{\Delta \quad p\text{H}} = 5.85\text{mg}/p\text{H}$ per g (Bate-Smith and Bendall, 1956) appears to hold true for the relaxed cod muscle in the present work, where $\frac{\Delta \text{ lactate}}{\Delta \quad p\text{H}} = 5.7$.

In summary, cod muscle relaxed at death, containing high initial levels of CP and ATP and very low levels of lactate, maintained its elevated level of ATP during iced storage to 24 hr postmortem; as a result, nucleotide catabolism, glycolysis, and onset of rigor were delayed. The thin-layer chromatographic procedure used proved a useful qualitative and semiquantitative tool for following the pattern of nucleotide degradation quickly and easily.

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Journal of the Fisheries Research Board of Canada

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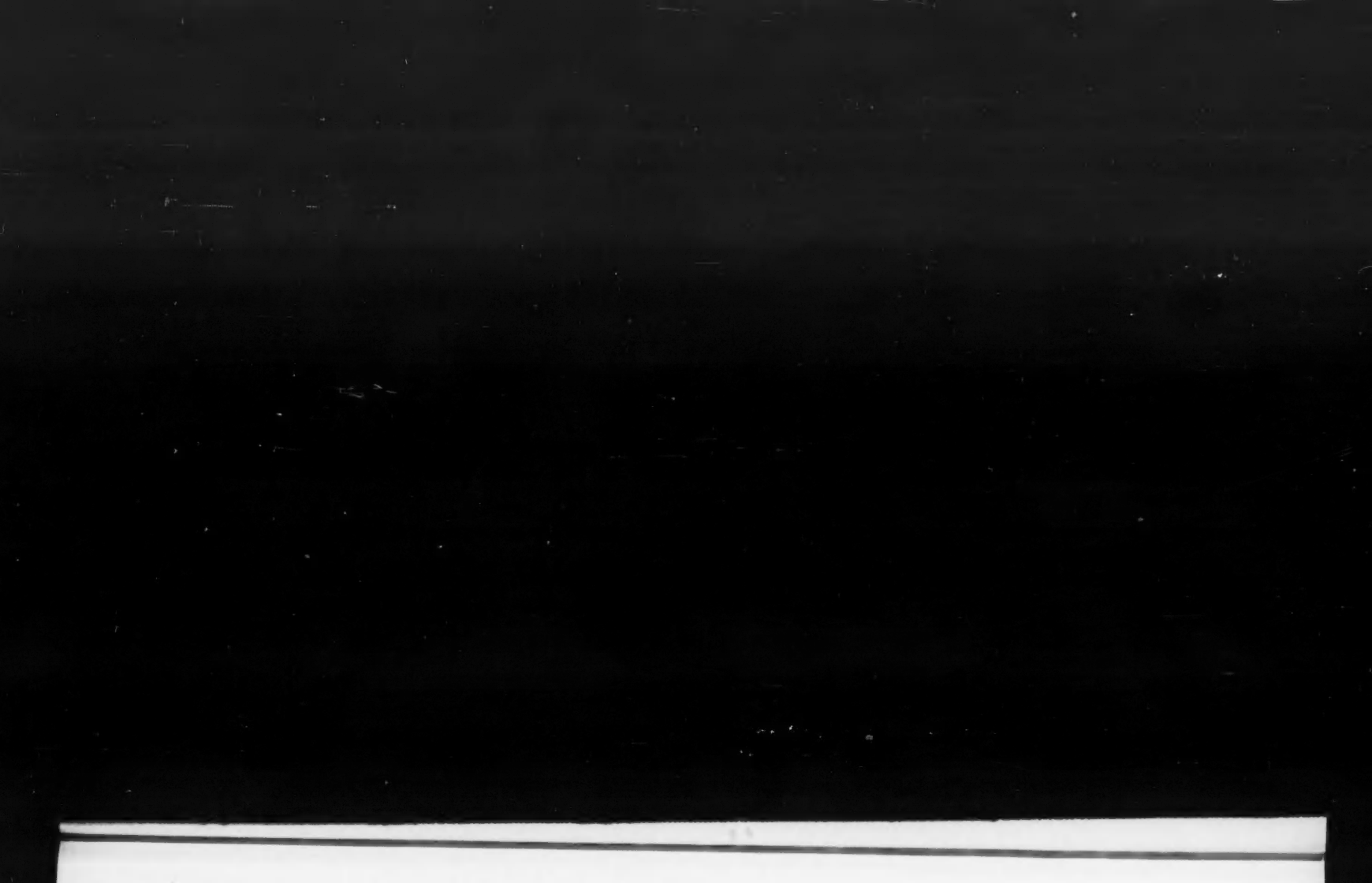
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